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Reduction of Group Reactivity of Complement Fixing Antigen of Meningopneumonitis Virus by Potassium Periodate.* (20697)

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Past attempts at demonstrating the specific antigen in viruses of the psittacosis-lymphogranuloma group have yielded negative or inconclusive results. In this laboratory several approaches have been tried including extraction of the antigen with solvents, differential centrifugation, and search for the specific component during the early phase of virus multiplication (before the appearance of the new mature virus). The preliminary reports by Barwell(1,2) suggested that potassium periodate could uncover the specific complement fixation antigen, presumably by virtue of destroying the group reactive "component". It was therefore decided to employ this substance in our investigations. The results of these studies agree in principle with Barwell's recently published observations(3). However, in our experiments it was found that the unmasking of the specific antigen was limited to certain conditions. Some of the results are presented in this paper.

Materials and methods. Virus and antisera. Meningopneumonitis (MP) virus and antisera were prepared as described in a previous communication(4). Human convalescent lymphogranuloma venereum (LGV) sera were obtained from patients. All sera were inactivated by heating at 56°C for 30 minutes. They were stored at −20°C. *Antigens.* Crude and purified antigens were prepared from infected allantoic fluid as described in the previous report(4). *Treatment with potassium periodate.* Virus was deposited in lusteroid tubes by centrifugation at 13000 RPM for 30 minutes. The

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TABLE I. Comparison of the Effect of KIO_4 on Various Preparations of MP Antigen.

Serum vs.	Antigen dil.	Antigen					
		Crude heated (Prep. 1)		Purified heated (Prep. 2)		Purified unheated (Prep. 3)	
		No KIO_4 treatment	Treated with KIO_4	No KIO_4 treatment	Treated with KIO_4	No KIO_4 treatment	Treated with KIO_4
MP	1:2	120*	20	40	<10	120	40
	1:4	120	20			40	P20
LGV	1:2	960	960	960	<30	1920	60
	1:4	960	960			960	<30

* Reciprocal of highest dilution of serum showing complete fixation.
P = Partial (2+) reaction.

sediments were resuspended in one-half the original volume of veronal buffer (pH 7.3) containing periodate in a final concentration of 0.005 M. On a few occasions, higher and lower concentrations of periodate were used but 0.005 M appeared to be most satisfactory. Action of periodate was allowed to proceed at 37°C for 30 minutes. Shorter time intervals were also employed but they gave less clear-cut results. The activity of periodate was neutralized by the addition of an amount of 5% glucose equal to the volume of periodate. A control preparation consisted of deposited virus suspended in a volume of veronal buffer (pH 7.3) equivalent to half the volume of starting material and also placed at 37°C for 30 minutes. An equal amount of 5% glucose was added to this virus suspension. Treatment with potassium periodate was carried out on four types of preparations: 1) Crude heated antigen. 2) Purified heated antigen obtained from 2 cycles of differential centrifugation. 3) Purified unheated antigen obtained from 2 cycles of differential centrifugation. 4) "Reconstituted crude" antigen. Representative findings obtained with preparations 1, 2, and 3 are shown in Table I.

Experimental. Effect of periodate on crude heated virus: (Preparation 1). Infected allantoic fluid was centrifuged at 1000 RPM for 10 minutes and the supernatant was heated at 100°C for 20 minutes. The heated preparation was then centrifuged at 13000 RPM for 30 minutes. The sediment was exposed to periodate in a manner described above. Periodate seemed to have *little or no effect when the antigen was tested with the heterologous system.* In the homologous system periodate brought about a 4- or 8-fold decrease in CF activity. Other experiments have consistently yielded similar results as regards the heterologous system. However, with the MP virus-MP antiserum combination, the results were irregular. Sometimes periodate reduced the activity of the crude antigen, other times it appeared to have no effect. The irregular decrease of CF activity observed in the homologous system cannot yet be explained.

Effect of periodate on purified heated antigen: (Preparation 2). Preparation was purified in the following manner: 20 ml of virus were centrifuged at 1000 RPM for 10 minutes and the resultant supernatant was centrifuged at 13000 RPM for 30 minutes in a Sorvall Angle centrifuge. The sediment was resuspended to the original volume in cold veronal buffer (pH 7.3) and the procedure was repeated. The sediment obtained from the second high speed centrifugation was resuspended in veronal buffer to the original volume and was heated at 100°C for 20 minutes. Treatment with periodate was as described under *Materials and methods.*

The results show a striking difference from those obtained with Preparation 1. Both preparations were strictly group reactive—the specific antigen having been destroyed by heating; yet the group reactivity of the first preparation persisted while that of the second preparation disappeared following treatment with KIO_4.

Effect of periodate on purified unheated antigen: (Preparation 3). Virus was washed by means of 2 cycles of differential centrifugation (same as preparation 2) and the final sediment treated with periodate in the manner described. The material was not heated and therefore contained, prior to treatment, both the specific and group reactive components. Following treatment, this preparation showed

TABLE II. Effect of KIO_4 on "Reconstituted MP Crude" Antigen.

				Antigen reconstituted	
Serum *vs.*	Antigen dil.	Purified heated antigen, control	Purified heated antigen + .005M KIO_4	in 1st & 2nd washing, heated, control	in 1st & 2nd washing, heated + .005M KIO_4
MP-hamster	Und.	80	20	80	80
	1:2	40	<20	40	20
	1:4	P40	—	<20	<20
LGV-human*	Und.	160	20	160	160
	1:2	80	<20	40	20 P40 P80
	1:4	P40	—	<20	<20

* Pool of sera from 3 patients.

no group reactivity or only a slight amount of such reactivity. It retained, however, a considerable amount of specific activity. Thus, with the unheated treated antigen preparation shown in Table I the heterologous serum reacted to only 3% or less of the original titre, while the homologous serum still exhibited a titre of 33% of the original.

Effect of periodate on "reconstituted crude" antigen: (Preparation 4). In one experiment, virus was washed as described in the previous sections. The final sediments were suspended either in a volume of veronal buffer or in a 1 to 1 mixture of the supernatants obtained from the first and second high speed centrifugations. Both preparations were heated and portions separated and treated with periodate in the following fashion: 9 ml of antigen were mixed with 1 ml of a 0.05 M periodate solution and the mixture incubated at 37°C for 30 minutes. The effect of periodate was stopped by the addition of 1.1 ml of a 50% glucose solution. The results in Table II compare the purified heated antigen with the "reconstituted crude" heated antigen. They show that while periodate had an effect on the purified preparation, it had little or no effect on this antigen when the purified virus was resuspended in the supernatants derived from the washing procedure. It would appear therefore that some undetermined component or factor associated with the viral antigen was shed in the washings. This is probably the component responsible for the resistance of the original crude heated preparation to the action of periodate.

Discussion. It was found that under certain conditions the group reactive "component" is susceptible to the action of periodate and can thereby be reduced or eliminated from an antigen of the MP virus. The specific component appeared to be fairly resistant. In order for KIO_4 to exert its full effect, the antigens had to be washed. The absorption experiments with crude and purified antigens(4), the presently recorded experiments on the effect of periodate on such preparations as well as studies now in progress on the effect of heat and phenol on CF activity of MP antigen preparations which were subjected to various procedures of washing and sonic vibration point to a heterogenous nature of the group reactive "component". It appears that the "component" is made up of at least one loosely bound constituent which is resistant to periodate and at least another constituent which is intimately bound to the virus particle and which is susceptible to periodate.

It is not clear at the present time whether the loosely bound component which is removed by washing and which is resistant to periodate is a naturally occurring group reactive constituent of the fresh antigen or whether it is artificially effected by the heating of the material with the resultant conversion of the specific component to a group reactive antigen or whether it is a gradually accumulating substance resulting from natural denaturation of the virus.

The effect of KIO_4 on antigens of other viruses of the psittacosis-lymphogranuloma group has not yet been determined in this laboratory.

Summary. It was found possible to reduce the group reactivity of the MP CF antigen by treatment with KIO_4. Three kinds of results were obtained depending on the state of the original antigen. 1) The group reactivity of the crude antigen was left practically unaltered. 2) Most of this reactivity was removed by KIO_4 from the purified antigen. Such preparations became inactive or only slightly active in the CF test with either homologous or heterologous antisera since the specific activity was previously destroyed by heating. 3) Antigens possessing the heat-labile specific component as well as the KIO_4-susceptible group-reactive "component" could be rendered fairly specific by treatment with KIO_4. It appears that the group reactive "component" is not a single entity, but is made up of at least 2 antigenic constituents.

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1. Barwell, C. F., *Nature*, 1948, v162, 460.
2. ———, *ibid.*, 1949, v164, 1013.
3. ———, *British J. Exp. Path.*, 1952, v33, 268.
4. Pollikoff, R., and Sigel, M. M., *J. Immunol*, in press.

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Adsorption of A_2isoagglutinogen-like Substances of Infectious Agents on Human Erythrocytes.[*] (20698)

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The A_2isoagglutinogen-like substance is the term applied to a polysaccharide complex which is either extracted from or secreted by animal parasites and bacteria. This substance is characterized by the following immunological properties: 1) The polysaccharide, as isolated chemically from the infectious agent, inhibits the α_2 isoagglutinins when added to human blood serums. The agglutinin titer in the serums against erythrocytes of Group A_2 (α_2 agglutinin titer), as observed at 37°C, becomes negative. There is also a slight inhibition of the α_1 isoagglutinins, but this may be due to the fact that the α_1 and α_2 antibodies may be partially bound together(1). 2) The polysaccharide is secreted when the living infectious agent is incubated in human serums. The titer of the α_2 isoagglutinins in the serums becomes negative. There is also a slight reduction in titer of the α_1, but no effect on the β isoagglutinins(2). 3) When the polysaccharide is injected intravenously into rabbits and monkeys, the titer of the α_2 isoagglutinins, as detected at 37°C, is reduced to negative for a period of 24 hours, and remains low for a period of 96 hours. The titer of the agglutinins acting at 6°C (cold agglutinins) against human and sheep erythrocytes, is also reduced after inoculation of the polysaccharide(3).

The term A_2isoagglutinogen-like substance seems therefore, appropriate, since the polysaccharide reacts specifically with the α_2 isoagglutinins in the serums. Studies on the possible relationship of the A_2isoagglutinogen-like substance with certain pathological conditions are being continued. Attempts were made to adsorb the polysaccharide onto human red blood cells to determine whether the A_2isoagglutinogen-like property could be transmitted to the erythrocytes.

Materials and methods. The erythrocytes utilized in this study were obtained from blood donors attending the Blood Bank of the School of Medicine, at San Juan, P. R. The red blood cells were washed at least 3 times with buffered saline (Bacto Hemagglutination Buffer, Difco) before using. Polysaccharides were extracted from dry and pulverized *Ascaris lumbricoides* from pig, larval forms of *Trichinella spiralis* from rat muscle, and adult *Taenia saginata*. The method of preparation has been reported previously(3). Glycogen, prepared in the same way, and rice starch, were used as controls. Ten mg of polysaccharide were suspended in 1 cc of buffered saline, to which 0.5 cc of packed erythrocytes was then added. The mixture was incubated at 37°C, or stored at 6°C, for one hour, with frequent shakings,

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TABLE I. Isoagglutinin Titers of Human Serums before and after Addition of Erythrocytes Treated with Various Polysaccharides.*

	RBC blood group	Group O serum				Group B serum			
		37°C		6°C		37°C		6°C	
		α_1	α_2	α_1	α_2	α_1	α_2	α_1	α_2
Before treatment		128	16	128	16	64	8	64	8
After treatment with polysaccharides:									
Ascaris	O	32	0	64	0	32	0	64	0
Ascaris	B	16	0	32	0	32	0	64	0
Trichina	O	64	0	64	0	8	0	64	0
Taenia	O	64	0	64	0	16	0	32	0
Glycogen	O	64	16	64	16	64	8	64	8
Corn starch	O	128	16	128	16	64	8	64	8
None	O	128	16	128	16	64	8	64	8
None	B	128	16	128	16	64	8	64	8

* The erythrocytes of human blood groups O and B in this particular test come from the same individuals. Similar results were obtained when each of 4 additional serums of Groups O and B was treated with adsorbed erythrocytes of 4 additional Group O and 4 Group B individuals.

followed by centrifugation at 4,000 rpm for 3 minutes. The supernatant was discarded; the sedimented erythrocytes being washed 3 times with buffered saline. After washing each time the suspension was allowed to sediment in 15 cc centrifuge tubes for 5 minutes, and the erythrocytes plus the free polysaccharide which settled to the bottom of the tube during the 5 minute period were discarded. In this manner assurance was made that all excess polysaccharide was removed, since none could be observed either macroscopically or under magnification at ×20. Furthermore, unseen smaller particles would be eventually washed off by the saline. This measure was taken in view of the fact that non-adsorbed polysaccharide remaining free in this suspension would have the same effect on the serums as the polysaccharide which would adsorb onto the erythrocytes. To 0.5 cc of the serums of various blood groups, 0.1 cc of the packed and carbohydrate-treated erythrocytes was added and incubated for 30 minutes at 37°C, with frequent shakings. The mixture was then centrifuged at 4,000 rpm for 3 minutes, the sedimented erythrocytes discarded, and the process repeated until the serum was treated 3 times with the polysaccharide treated erythrocytes.

Serums were also treated with erythrocytes to which no polysaccharide had been adsorbed, as control. The serums were then tested for their content of α_1, α_2, and β isoagglutinins. For these tests 0.1 cc volumes of the serums in Wassermann tubes were diluted 2-fold with buffered saline. An equal volume of a 0.5% suspension of the erythrocytes was added. Agglutination of the erythrocytes was observed under the binocular microscope, at a magnification of ×20, after incubation at 37°C for one hour, and after storage at 6°C overnight.

Results. The polysaccharides from animal parasites seemed to have adsorbed onto the erythrocytes without difficulty. Treatment of the erythrocytes with the polysaccharide suspension, either at 37°C or 6°C, always gave identical results.

Inhibition of α_2 isoagglutinins by erythrocytes onto which the polysaccharides had been adsorbed. Table I presents the results of a typical experiment. Erythrocytes of human blood groups O and B, after treatment with polysaccharides extracted from the various animal parasites, inhibited the α_2 agglutinins acting at 37°C and 6°C. The erythrocytes from 5 individuals of blood group O and 5 from blood group B were each treated with the polysaccharides and each used to inhibit the agglutinins in 5 group O and 5 group B serums. In all instances the titers of the α_2 isoagglutinins were reduced to zero. Inhibition of the α_2 isoagglutinins must be attributed to the polysaccharide adsorbed onto the erythrocytes, since in the first place, none could be observed free in the suspension after the cells were washed, and second, since as previously reported, the amount of free polysaccharide required to inhibit the isoagglutinins is in the order of 20 mg per 0.5 cc which is double the amount utilized for the suspension into which the erythrocytes were added(1).

The titer of the α_1 was also reduced as compared with that of the serums treated with the erythrocytes without polysaccharide, but were not brought down to zero, as observed with the α_2 isoagglutinins. The titers of the β isoagglutinins were unaffected. No significant change in titer of all agglutinins was observed when the serums were treated with erythrocytes free of polysaccharide.

The erythrocytes treated with glycogen and corn starch did not inhibit the α_2 isoagglutinins, either at 37°C and 6°C, as observed with the erythrocytes treated with the polysaccharides from the animal parasites.

Discussion.. The results of the above studies suggest that polysaccharides isolated from various species of animal parasites adsorbed onto human erythrocytes of groups O and B. Erythrocytes of blood groups A_1 and A_2 were not utilized, since these necessarily would inhibit the α_2 isoagglutinins by virtue of their homologous or related agglutinogens. The treated erythrocytes acquired characteristics of human blood group A_2, since in the serums into which they were introduced, the α_2 isoagglutinin titer was reduced to zero. No significant change in the titer was observed when the serums were treated with non-adsorbed erythrocytes.

That erythrocytes of a certain blood group can acquire characteristics of another, when polysaccharides from infectious organisms adsorbed onto their surfaces, is a finding of great interest which may have important implications. As previously pointed out, the polysaccharides *per se* have A_2isoagglutinogen-like properties, and also the infectious organisms secrete the substance when incubated in serum. During infection with organisms secreting the A_2isoagglutinogen-like substance, the erythrocytes of the host may become coated with the polysaccharide. This would introduce new antigenic groups onto the erythrocyte pattern and thus the host may produce antibodies against the new erythrocyte-polysaccharide complex and therefore against its own erythrocytes. Such antibodies may agglutinate the host erythrocytes if they are of the A_2 group, or of any other blood group which may have the polysaccharide adsorbed onto them.

Autoagglutination and hemolysis of erythrocytes, as observed in blackwater fever and other conditions in which infectious agents are involved, may be associated with such a phenomenon by which erythrocytes become coated with polysaccharides related to blood substances.

Summary. 1. Partially purified polysaccharides isolated from dry worm material from adult *Ascaris lumbricoides* (*var suum*); the larval forms of *Trichinella spiralis* and adult *Taenia saginata* adsorbed onto human erythrocytes of groups O and B, when these were incubated at 37°C or stored at 6°C in saline suspensions of the polysaccharide. 2. The treated erythrocytes were added to human serums of groups O and B and the α_2 isoagglutinin titers were reduced to zero. No significant reduction of the α_2 isoagglutinin titer was observed when control erythrocytes were added to the serums. 3. The titers of the α_1 was slightly reduced and that of the β isoagglutinins was unaffected. 4. The fact that the erythrocytes treated with the polysaccharides inhibited the α_2 agglutinins is used as evidence to show that the polysaccharides adsorbed onto the red cells. 5. Adsorption of polysaccharides with A_2 isoagglutinogen-like properties, onto erythrocytes of human groups O and B gives the cell characteristics pertaining to a different group, *i.e.*, the A_2 group. The possibility of such phenomenon occurring during actual infection, and the relationship which it may have with the development of autohemagglutinins is mentioned.

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1. Oliver-Gonzalez, J., *J. Infect. Dis.*, 1944, v74, 173.

2. ———, *ibid.*, 1949, v85, 66.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 559.

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Phosphorus Metabolism in the Dog Kidney. (20699)

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The experimental methods used to study metabolic processes of individual organs are of three general types: 1) use of tissue slices and homogenates *in vitro*; 2) perfusions of isolated organs *in vitro* or *in vivo*; and 3) tissue fractionations and analyses after the injection of metabolites or labelled materials into the intact animal. Although these methods have yielded valuable information, they have some serious shortcomings. Thus, the condition of tissue slices and homogenates is far from physiological and, in many cases, the amount of various metabolites which must be added in order to obtain a recognizable metabolic reaction is far greater than that existing *in vivo*. Furthermore, in most experiments of this type, no provisions are made for the removal of the products of metabolic reactions and the system works without the intervention of the diffusion and osmotic phenomena which play an important role in the kinetics of an intact biological system. The liquids used in perfusion experiments, in most cases, are not physiological since, on the one hand, they do not contain necessary metabolites and hormones while, on the other hand, a given metabolite may be added in amounts far in excess of the normal, bringing into play concentration effects and thus exaggerating reactions which normally may be negligible. Tracer experiments also have important limitations consisting, as they generally do, of the injection of a labelled material, sacrifice of the animal after a given period of time, and isolation of the tracer element. This method is adequate to study the overall metabolism or the metabolism of one organ within the framework of the general economy of the body, but may not be sufficient if one wishes to examine the individual contribution of a single organ to the metabolism of the organism as a whole.

In a normal animal, all organs are in a steady state not only within themselves, but also in respect to each other and when the metabolism of one organ becomes abnormal, other organs may be affected by the changes in the amounts of critical materials which are caused by the primary disturbance. The secondary phenomena may, indeed, overshadow the primary ones and tracer technics may not distinguish between the two. The interdependence of the endocrine glands offers many pertinent examples which are of practical as well as of theoretical interest. For these reasons, a more physiological method was sought.

Experimental. The method consists of injecting a tracer material into the intact artery of an organ while the blood emerging from the organ is collected in a beaker by means of a venous cannula. This technic prevents the blood leaving the organ under investigation from returning to it after having circulated elsewhere. It is, in effect, a "single pass" experiment which combines the advantages of a sensitive tracer technic with those of leaving an organ *in situ,* perfused by its own normal blood supply, and yet avoiding all contact of the tracer element with other organs of the body. In view of the fact that the blood vessels of the kidney are readily accessible, this organ was chosen for our studies. Healthy, medium-sized, mongrel dogs of both sexes were fasted for eighteen hours and anesthetized by means of an intraperitoneal injection of Na pentobarbital (35 mg/kg). One kidney was exposed and completely isolated except for the main renal vessels. After the dissection was completed, the dog was heparinized (2 mg/kg I.V.),[†] the renal vein cannulated and the blood allowed to flow into a beaker for about 1 minute. Fifty to 100 microcuries of P^{32} as sodium phosphate (10-20 μg of phosphorus)[‡] dissolved in about 10 ml of physiological saline

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[†] Heparin was donated by Wilson Laboratories, Chicago, Ill.

[‡] P^{32} was supplied by Abbott Laboratories on allocation from U. S. Atomic Energy Commission.

TABLE I. Concentration of P in TCA Soluble Fractions of Dog Kidney. (mg P/100 g wet tissue.)*

Dog No.	1		2		3		4		5	
Fraction	C	M	C	M	C	M	C	M	C	M
Inorganic	18.6	—	22.2	18.3	31.5	20.1	27.0	18.0	30.3	18.9
ADP-ATP	2.34	—	2.07	1.44	3.60	1.14	—	2.00	1.51	.84
G-1-P	.27	—	.94	1.44	.90	1.20	.75	.58	.70	1.26
G-6-P	—	—	—	—	1.48	2.46	2.10	2.66	.46	1.05
Coenzyme	1.14	—	—	4.6	6.75	1.83	1.65	5.66	1.08	3.78

* C = cortex, M = medulla, ADP-ATP = labile P of ADP-ATP, G-1-P and G-6-P = glucose-1 phosphate and -6 phosphate, respectively.

were injected into the main renal artery and were thus carried into the kidney by the normally flowing arterial blood. The injection lasted for about 1 minute and was followed by a 1-3 minute period during which time the P^{32} remaining in the blood vessels of the kidney was flushed out by the supervening normal arterial blood. The venous blood, containing the excess P^{32}, was collected into a beaker and thus was not allowed to recirculate. The total time during which the renal blood was allowed to flow to the outside was 3 to 5 minutes; the total blood lost was 150 to 200 ml. This allowed the blood pressure to remain well above shock levels, as determined in a number of preliminary experiments. At the end of the perfusion period, the kidney was rapidly removed, chilled by injecting ice cold saline through the venous cannula (thus washing out most of the blood remaining in the organ), and cut longitudinally. Within 1 or 2 minutes, samples of cortical and medullary tissue were frozen in powdered dry ice. The parts which could not be well separated were discarded. The frozen tissue was weighed, dropped into 50 ml of ice cold 10% trichloracetic acid (TCA) and homogenized with a Waring blender in the cold room. The temperature of the room was about 1°C and that of the homogenate was kept below 5°C by the addition of small pieces of dry ice. The suspension was centrifuged in the cold room and the solids re-extracted 3 or 4 times with 25 ml of ice cold 5% TCA. This was sufficient to extract at least 95% of the total activity. The specific activity of the residue, containing phosphoprotein and phospholipid, was negligibly small and was not examined in detail. The volume of TCA extracts was measured and duplicate 40 ml aliquots were used to fractionate the phosphorus into the following fractions: inorganic-P, coenzyme-P, glucose-1-P (G-1-P), glucose-6-P (G-6-P), and the labile phosphorus of adenosine di- and triphosphate (ADP-ATP). The fractionation was done according to the method of Sacks (1). Phosphorus was determined according to Fiske and SubbaRow(2). Radioactivity was measured as follows: when necessary, each fraction was oxidized with nitric acid to inorganic phosphate and diluted to 10.0 ml. A suitable aliquot was used to determine phosphorus quantitatively. To another aliquot 1.0 mg of inorganic phosphate was added as carrier and the phosphorus was precipitated as ammonium phosphomolybdate. The precipitate was filtered through small circles of filter paper mounted on a stainless steel suction funnel,§ washed, dried, and its radioactivity measured by means of a thin window Geiger Muller tube. The error of counting was 3% or less.

Results. Table I shows that in all cases the concentrations of inorganic-P and of ADP-ATP were greater in the cortex than in the medulla. On the other hand, the concentrations of G-1-P, G-6-P and coenzyme-P tended to be greater in the medulla than in the cortex. The notable exception is animal No. 3 in which a larger amount of the coenzyme-P was found in the cortex. We are unable to explain this apparently aberrant result, as the only detectable difference between animal No. 3 and the others was the fact that this animal required a double dose of nembutal for anesthesia. The values obtained fall within the range of those reported by Dratz and Handler (3) for the whole cat kidney.

Table II shows that the specific activity of all fractions is greater in the cortex than in

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§ Tracerlab, Inc., Boston, Mass.

TABLE II. Specific Activities of TCA Soluble Fractions of Dog Kidney. (Counts/min./mg P) × 10^{-3}.

Dog No.	1		2		3		4		5	
Fraction	C	M	C	M	C	M	C	M	C	M
Inorganic	70.5	—	89.9	27.5	391	213	45.6	37.0	530	240
ADP-ATP	45.2	—	61.3	15.6	131	110	—	23.2	484	183
G-1-P	45.6	—	24.4	3.4	146	20	30.4	7.9	248	96.6
G-6-P	—	—	—	—	6.6	1.66	1.44	.69	11.4	14.4
Coenzyme	22.6	—	—	2.84	47	2.73	4.85	1.39	140	15.7

TABLE III. Specific Activities of TCA Soluble Fractions of Dog Kidney Divided by Specific Activity of Inorganic P.

$$\left(\frac{\text{S. A. bound P}}{\text{S. A. inorganic P}}\right)$$

Dog No.	1		2		3		4		5	
Fraction	C	M	C	M	C	M	C	M	C	M
ADP-ATP	.64	—	.68	.57	.33	.51	—	.62	.91	.76
G-1-P	.65	—	.26	.12	.37	.09	.67	.21	.47	.40
G-6-P	—	—	—	—	.02	.01	.03	.02	.02	.06
Coenzyme	.43	—	—	.10	.12	.01	.11	.04	.26	.06

the medulla. In both tissues, the specific activities decrease in the following order: inorganic-P, ADP-ATP, G-1-P, coenzyme-P, G-6-P.

Discussion. Individual results vary from experiment to experiment. This is probably due to differences between individual experiments such as the rate of injection of the isotope, the time elapsed before freezing the tissues or the amount of blood flowing through the kidney, as affected by anesthesia, visceral manipulation and variable blood loss. The fact that the specific activities of all fractions are higher in the cortex than in the medulla indicates a greater turnover in the former than in the latter, which is in agreement with the fact that the cortex has a more active metabolism than the medulla(4,5). When the data are calculated in terms of specific activity (SA) of inorganic-P, some of the differences between cortex and medulla are not as large (Table III).

The high relative specific activity for G-1-P in the cortex indicates a very high turnover rate, in some cases as high or higher than that of ADP-ATP. G-1-P can be formed in three ways(6): 1) from glucose-6-phosphate; 2) directly from glucose through a reaction similar to that described for galactose; and 3) from glycogen and inorganic-P via the phosphorylase reaction. The first two possibilities do not appear likely because the specific activity of G-1-P is much higher than that of the would-be precursors, G-6-P and ADP-ATP. G-1-P must, therefore, be formed predominantly via the third reaction.

When a steady state exists, the rate of formation of a substance must be equal to its rate of disappearance. Three possible manners of G-1-P disappearance have been suggested (7): 1) conversion to glycogen through a reversal of the phosphorylase reaction; 2) conversion to G-6-P; or 3) conversion to glucose through a reversal of the glucose-phosphokinase reaction. The first alternative is possible despite the low glycogen content of the kidney (8). The second possibility appears unlikely because the difference between the activity of G-1-P and that of G-6-P is too great. Sacks (9) has shown that also in the liver G-1-P is not a likely precursor of G-6-P. The third possibility has not been investigated as yet.

The high concentration and low specific activity of the G-6-P in renal tissue seems to indicate that its synthesis in the kidney is insignificant and that a large part of it is synthesized elsewhere and carried by the arterial blood to the kidney to be stored and used there. This seems possible since the blood cells contain 50 to 100 mg of acid soluble phosphorus per 100 ml(10), 2.72 mg of which appears to be G-6-P(11), both values being similar to those found in kidney tissue. In view of the high overall metabolic activity of kidney tissue, the low S.A. of G-6-P would indicate that, in the kidney, this compound may not occupy a central position in the metabolism of carbohydrate.

Conclusions. 1. A "single pass" technic is described for studying the intrinsic metabolism of the kidney. 2. It is shown that P uptake by ADP-ATP and G-1-P is very rapid, more so in the cortex than in the medulla. G-1-P probably is derived from glycogen via the phosphorylase reaction. 3. The very low relative S.A. of the G-6-P seems to indicate that most, if not all, of the G-6-P found in the kidney is formed elsewhere in the body and carried to this organ by the blood.

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Antagonism of Chemotherapeutic Activity of p-Aminosalicylic Acid in Experimental Tuberculosis by p-Aminobenzoic Acid.* (20700)

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In vitro antagonism of the tuberculostatic properties of para-aminosalicylic acid (PAS) by para-aminobenzoic acid (PABA) has been established(1-3). The *in vivo* relationship of PAS to PABA has not been reported. The purpose of this communication is to present the results of an investigation of the effect of dietary PABA on the chemotherapeutic activity of PAS in experimental tuberculosis.

Methods. The H37Rv strain of *M. tuberculosis* was utilized as the infectious agent in this study. Male CF_1 mice weighing 14 to 16 g each were employed as test animals. The basal ration represented a modification of a synthetic ration devised by Pearce *et al.*(4). Its composition was as follows: vitamin-free casein, 20%; Wesson's salt mixture(5), 4%; Ruffex, 10%; corn starch, 55.68%; sucrose, 5%; lard, 5%; and supplement, 0.313%. The following components of the supplement are expressed in terms of mg per 100 g of basal ration: pyridoxine hydrochloride, 1.0; nicotinic acid, 1.0; thiamine chloride, 0.6; riboflavin, 0.6; calcium pantothenate, 2.0; folic acid, 0.05; biotin, 0.05; PABA, 30; inositol, 50; choline chloride, 100; cystine, 100; alpha tocopherol, 3.5; and menadione, 2.5. Concentrations of 1,500 I.U. of vit. A and 200 I.U. of vit. D were added per 100 g basal ration. Adjustment of the concentrations of PABA and PAS in the rations was accomplished by the addition or omission of equal weights of starch. Five rations were studied in Exp. A and were as follows: 1) basal ration; 2) basal ration, PABA-free;‡ 3) basal ration containing 2% PABA; 4) basal ration containing

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‡ The term, "PABA-free" refers only to omission of PABA from the supplement.

TABLE I. Comparison of Weights and Survival Times of Tuberculous Mice Receiving a Basal Ration and Rations Containing Varied Amounts of PAS with and without PABA.

Rations	Avg wt mice (g) A*	B*	C*	T_{50} and confidence limits	% surviving on 55th day
A. B.R.†	13.1	18.2	13.9	16.0 (14.0–18.0)	0
”	13.7	19.8	13.5	18.7 (17.0–20.6)	0
” + 2% PABA	13.9	15.0	12.4	15.6 (13.8–17.5)	0
” + .5% PAS	13.4	19.2	15.5	0‡	69.6
” + 2% PABA + .5% PAS	13.7	13.0	11.2	21.4 (18.8–24.6)	0
B. B.R.	16.2	18.4	12.6	16.3 (14.3–18.6)	0
” + .125% PAS	16.7	17.8	11.9	25.0 (22.5–30.0)	0
” + .25% PAS	16.0	18.3	11.5	35.0 (30.6–40.0)	0
” + 1% PABA + .125% PAS	15.6	17.2	11.9	19.4 (16.6–22.7)	0
” + 1% PABA + .25% PAS	14.8	15.7	10.9	24.3 (22.4–26.4)	0

* A—Wt of mice on arrival. B—Wt of mice at inoculation. C—Wt of mice at death.
† B.R. = Basal ration.
‡ T_{50} was not attained.

0.5% PAS, PABA-free; 5) basal ration containing 2% PABA and 0.5% PAS. In Exp. B the following rations were included: 1) basal ration; 2) basal ration containing 0.125% PAS, PABA-free; 3) basal ration containing 0.25% PAS, PABA-free; 4) basal ration containing 1% PABA and 0.125% PAS; 5) basal ration containing 1% PABA and 0.25% PAS. Each ration was furnished *ad libitum* to a group of 20 mice. In Exp. A and B prefeeding periods of 8 and 5 days respectively, were instituted prior to injection of organisms. The animals were infected by intravenous injection of 0.15 mg (dry weight) of *M. tuberculosis*. The organisms had been cultured according to the method of McKee *et al.*(6). The survival time of each animal was recorded in terms of days following injection of organisms. The mortality rate for each group was plotted on an arithmetic-probability scale from which the 50% survival time (T_{50}) was determined (7). The death-rate response of each test group was examined statistically by the method of Litchfield(8).

Results. The data of Exp. A presented in Table I suggested antagonism of the tuberculostatic properties of PAS by orally-administered PABA. The results of this experiment did not permit definite conclusions since the concentration of PABA in rations furnished Groups III and V proved to be toxic as evidenced by the retarded gain in weight of the animals. However, a comparison of the T_{50} of Groups I and III indicated that the toxicity of the ration containing 2% PABA did not increase the susceptibility of the mice to experimental tuberculosis significantly. This finding tended to support the validity of the difference in percentages of mice surviving in Groups IV and V at the termination of the experiment.

In Exp. B the reduction of the concentration of PAS in the rations yielded a suitable T_{50}. Reduction in concentration of PABA resulted in minimal toxicity. The data of Exp. B illustrate antagonism of the tuberculostatic activity of PAS by PABA. The chemotherapeutic activity of PAS was evidenced by the increased T_{50} of the animals in Groups II and III as compared with those in Group I. Antagonism of the tuberculostatic properties of PAS by dietary PABA was likewise apparent in the reduced T_{50} of the animals in Groups IV and V as compared with the T_{50} of those in Groups II and III.

Under natural conditions it is doubtful if the dietary intake of PABA in man or animals ever approximates the amounts employed in this study. Among foodstuffs, yeast has been found to be one of the richest sources of PABA, containing up to 100 μg per g and of which 70 to 80% is present in the free form(9). The addition of such a foodstuff to the diet would result in a concentration of PABA much less than 1%. Denko *et al.*(10) have provided evidence that PABA is synthesized by microorganisms in the intestine of man and thereby made available to the host. These investigators have reported that the urinary and fecal excretion of PABA may exceed the dietary intake by approximately 100%. However, since the average daily urinary and fecal ex-

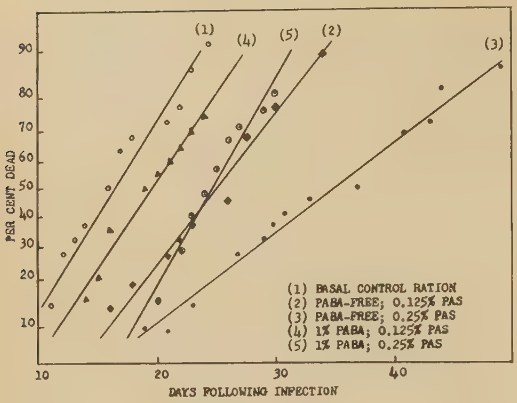

FIG. 1. Rate of death of tuberculous mice fed basal ration and rations containing varied amounts of PAS with and without PABA.

cretion was 737 μg the amounts involved were relatively small. The excretion of PABA in the urine and feces of the mice used in this study was not determined.

In studies of the relationship of PABA to the sulfonamides in the metabolism of *E. coli* and other organisms, it has been shown that the observed inhibition index SA/PABA(11, 12) may be progressively increased in the presence of end-products of the inhibited reaction(13,14). If such a relationship should exist with respect to PAS and PABA in the metabolism of *M. tuberculosis*, the presence of end-products of the inhibited reaction might render relatively small amounts of PABA effective in antagonizing the chemotherapeutic activity of PAS.

McKee *et al.*(6) reported that the H37Rv strain of *M. tuberculosis* was unsuitable for therapeutic studies in CF_1 mice since the animals did not die at a uniform rate under conditions which yielded extended survival. Youmans and Youmans(15) found, however, that infection of the "Strong A" strain of mice with H37Rv resulted in a uniform rate of death of the animals providing a sufficiently large infecting dose was used and that the uniform response was maintained under conditions of therapy in which the T_{50} was extended. The data presented in Fig. 1 indicate that under the conditions of the present study in which a limited number of animals were used, infection of CF_1 mice with H37Rv results in rates of death which yield approximately straight lines when plotted on probability paper indicating a normal frequency distribution curve.

Summary. 1. Infection of CF_1 mice with the H37Rv strain of *M. tuberculosis* produced a uniform response in rate of death. 2. Antagonism of the chemotherapeutic activity of PAS in experimental tuberculosis by dietary PABA was demonstrated.

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Effects of Injection of 5-Adenylic Acid on Tissue Nucleotides of the Rat.
(20701)

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Intramuscular injection of 5-adenylic acid in man produces significant shifts in the different nucleotide fractions present in the red cells, but no significant variation in the total nucleotide with doses between 20 and 100 mg(1). The present paper reports data on the alteration of adenine nucleotide levels in brain, muscle, liver, heart, and kidney of the rat in control animals and in animals injected with 5-adenylic acid and the localization of 5-adenylic acid labelled with radioactive phosphorus in various tissues.

Procedure. Adult, white, male rats weighing between 125 and 500 g were employed. No significant differences were observed with different batches of rats. The animals were injected with either 10 or 100 mg of 5-adenylic acid as the sodium salt,* intraperitoneally. Approximately 50 minutes after injection and 10 minutes before sacrifice, the animals were anesthetized with nembutal intraperitoneally (5 mg/100 g body weight). One hour after nucleotide injection, the skin surrounding the gastrocnemius muscle was carefully dissected free. The connective tissue sheath around the muscle was cut and the blunt end of a scalpel inserted beneath the connective tissue sheath freeing the muscle but allowing it to remain attached at its origin and its insertion. The animal was allowed to recover from the shock of the operation for 10 minutes, at which time the head was immersed in liquid nitrogen. While the skull was freezing, the gastrocnemius muscle was rapidly removed and transferred to liquid nitrogen. The time required for this manipulation varied between about 10 and 30 seconds. After the skull was frozen, the brain was removed without being allowed to thaw and also immersed in liquid nitrogen. Brain and muscle were then pulverized and deproteinized with trichloracetic acid. Liver, kidney, and heart, in the earlier experiments, were removed in the frozen state after the entire animal was frozen in liquid nitrogen. In later experiments, the tissues were removed from anesthetized animals and immediately plunged into liquid nitrogen. The results obtained with these two procedures were not significantly different. These tissues were also pulverized and deproteinized with trichloracetic acid. Tissues from control animals not receiving the 5-adenylic acid were treated in similar fashion. In the case of brain and muscle, the protein-free trichloracetic acid extracts were immediately analyzed for inorganic orthophosphate and phosphocreatine phosphorus according to the procedures described by LePage, in Umbreit, Burris, and Stauffer(2). The trichloracetic acid extracts remaining after carrying out these determinations were treated with barium and alcohol. The barium-alcohol insoluble precipitates resulting were collected by centrifugation, freed of barium and assayed for Adenylic Acid (AA), Adenosine Triphosphate (ATP), and Adenosine Diphosphate (ADP), enzymatically according to the procedure of Albaum and Lipshitz(3). Total purine was also calculated as adenine from the absorption in the Beckman spectrophotometer at 2600 Å. Some blood nucleotide determinations were also carried out on separate animals following the injection of 5-adenylic acid intraperitoneally. The blood was removed under heparin by cardiac puncture. AA, ADP, ATP, and total purine were measured on perchloric acid filtrates prepared as described previously(1). For the *tracer experiments,* adenylic acid with the phosphorus labelled as P^{32} was prepared by a fermentation procedure. The injected dose, 50 mg, contained 784,000 counts/minute. This was injected intraperitoneally and the animals were sacrificed under anesthesia at the end of one hour.

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* Supplied as My-B-Den by the Ernst Bischoff Co. Inc., Ivoryton, Conn.

TABLE I. Phosphocreatine Levels of Brain and Muscle and Adenine Nucleotide Levels of Brain, Muscle, Heart, Liver, and Kidney Following Injection of 100 mg 5-adenylic Acid Intraperitoneally. (Animals sacrificed 1 hr after injection; adenine nucleotide levels expressed as mg % adenine; all values are mean values.)

	Brain		Muscle		Heart		Liver		Kidney	
	C (39)†	E (19)	C (41)	E (20)	C (11)	E (9)	C (11)	E (8)	C (12)	E (9)
PC	50.0	70.8*	218.2	264.5*						
AA	5.4	3.9*	2.1	1.1	13.3	10.9	11.4	11.3	9.5	9.7
ADP	1.4	1.1	1.2	2.4	5.5	4.6	5.9	4.6	3.5	3.3
ATP	14.2	21.8*	42.5	54.5*	32.6	41.1*	14.7	26.6*	12.0	11.7

* Difference between mean values of Control (C) and Injected (E) statistically significant. Statistical significance concluded if difference between means exceeds S.E. diff. by more than 2×. S.E. diff. $= \sqrt{(\text{S.E.}_C)^2 + (\text{S.E.}_E)^2}$.

† The figures in parentheses indicate the No. of different analyses performed.

TABLE II. Radioactivity in Various Tissues Following Injection of 50 mg of 5-adenylic Acid Intraperitoneally. (Animals sacrificed at the end of 1 hr.) Total counts/min. injected = 784000.

Tissue	Counts/min./mg* fresh wt
Adrenal	337
Liver	285
Kidney	195
Heart	145
Spleen	98
Blood	52
Testis	16
Muscle	16
Brain	11

* Each value is avg of 6 determinations.

TABLE III. Total Purine Content of Various Tissues Following Injection of 100 mg 5-adenylic Acid Intraperitoneally. (Animals sacrificed 1 hr after inj.; total purine values calculated as mg % adenine from 2600 Å reading.)

Tissue	n	Control	n	Exp.
Brain	7	35.8	21	39.3
Muscle	7	55.8	21	60.4
Heart	10	76.1	9	76.0
Liver	10	66.1	9	81.8*
Kidney	10	48.1	9	48.8

* Difference between means statistically significant.

On removal, the tissues were dissolved in 30% KOH and aliquots were transferred to planchets. These were dried and counted.

Results. After the injection of 10 mg, the only statistically significant result was a lowering of the AA of both muscle and brain. 100 mg of 5-adenylic acid, on the other hand, produced a marked increase in the adenosine triphosphate and phosphocreatine in muscle and in brain, without any significant change in the total purine (Tables I, III). Increases in ATP of human blood without alteration of the total purine level have been shown previously(1).

This indirect effect of the injected nucleotide on the purine "reservoir" prompted us to investigate the distribution of adenylic acid labelled with radioactive phosphorus in other tissues. The results are shown in Table II. On a weight basis, the adrenal gland concentrates the isotope more than any other tissue, followed by liver, kidney, and heart. Brain and muscle accumulate only small quantities.

Because of these results, we carried out nucleotide analyses on liver, kidney, and heart. Table I shows that there was no effect on the kidney nucleotides of injected 5-adenylic acid which suggests that the tracer is probably in the process of being eliminated. Liver and heart, however, showed significant increases in their ATP level (Table I). One point merits special emphasis: the heart showed no increase in total purine so that the alterations observed probably represent shifts in a nucleotide "reservoir". On the other hand, liver shows an increase in total purine which is statistically significant (Table III).

TABLE IV. Blood Nucleotides in the Rat. Mean values as mg % adenine.

	Control (n = 12)	100 mg AA inj.† (n = 16)
AA	.2	.7*
ADP	.7	.6
ATP	4.1	5.1*
Total purine	8.9	10.6*

* Difference between means statistically significant.

† Blood drawn ½ to 1 hr after inj.

The results on the rat blood removed after one hour are shown in Table IV. The concentration of known nucleotides and total purines is lower in rats than it is in mice and in men. The injection of adenylic acid produces a small but significant increase in the adenosine triphosphate level of the rat blood, and a corresponding increase in the total purine. Whether this difference between the behavior of the blood of rat and man is related to some peculiarity of the rat or whether this is related to the dose of adenylic acid injected, is not clear at the present time.

Discussion. On the basis of our original blood work(1), we had concluded that the effect of injected 5-adenylic acid on the blood was not a direct one, since the magnitude of the effect observed was, in most cases, in excess of what might be expected on the basis of the amount of adenylic acid injected. The fact that we have also observed an increase in the phosphocreatine level in brain and in muscle supports this conclusion. The conclusion is also supported by experiments in which it has been demonstrated that injected adenylic acid lowers the cholesterol levels, and produces alterations in the serum lipoprotein of experimental animals(4,5). All of these diversified effects produced by the injection of the nucleotide make it appear that the effect is not a simple one, but is rather complex. This, coupled with our observation that the adrenal gland accumulates radioactive phosphorus from injected adenylic acid in much greater concentration than any other tissue, opens some interesting possibilities with respect to this organ being implicated in some of the observed effects.

As our data show, not all organs show alterations in tissue nucletoide on the injection of 5-adenylic acid; for example, kidney. Certain tissues like tumors(6), on the other hand, as we reported earlier, show a significant decrease in nucleotide.

Summary. 1. Tissue nucleotide levels (AA, ADP, ATP, and total purine) of control rats and those injected with 5-adenylic acid have been studied. At the 100 mg injection dose, brain, muscle, liver, and heart show significant increases in adenosine triphosphate. Of these 4 tissues, only liver exhibits a measurable increase in total purine. Kidney shows no effect. The cellular element of blood shows a small but significant increase in adenosine triphosphate, but this is accompanied by a corresponding increase in total purine. 2. The experiments in which 5-adenylic acid, containing radioactive phosphorus is injected into rats shows a maximum accumulation of the label on a weight basis in the adrenal gland followed by liver, heart, kidney, spleen.

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Analgetic Activity of Optical Isomers of 3-hydroxy-N-methylmorphinan* and 6-dimethylamino-4,4-diphenyl-3-heptanone.†‡ (20702)

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Although many potent analgetics have resulted from investigation of this class of pharmacologic agents, their mechanism(s) of action has not been elucidated. It is generally accepted that the morphine-type analgetics possess a similar mechanism of action, although no direct evidence in support of this concept is available. Several investigators(1-7) have demonstrated that the *l*-isomers of optically active analgetics possess all or most of the analgetic activity of the racemic form of the compound. Slomka and Gross(4) demonstrated that dextrorphan tartrate inhibited the analgetic activity of levorphan tartrate and of morphine sulfate. This indicated that these structurally very similar compounds possessed a similar mechanism of action. It was of interest to extend this study to a structurally somewhat dissimilar analgetic agent in order to test the accepted but unproven contention that the mechanism of action of the morphine-type analgetics is the same.

Methods. The effects of drug treatments were determined on 6 trained normal human subjects using a warm wire analgesiometer patterned after that of Lee, Williams, and Pfeiffer(8). Pain thresholds were determined on the finger nail bed at 30-minute intervals. The subjects were allowed freedom of movement during the experimental period. The minimal intensity increment that the subjects were required to distinguish was equivalent to approximately a 4% rise in threshold, and this represents the maximal variability of the apparatus. The drugs in physiologic saline, were administered subcutaneously. The treatments tested were: 1) 2.0 mg levorphan tartrate; 2) 3.2 mg levorphan tartrate; 3) 3.2 mg dextrorphan tartrate; 4) 3.2 mg levorphan tartrate and 3.2 mg dextrorphan tartrate; 5) 3.2 mg levorphan tartrate and 5.0 mg *d*-Methadone Hydrochloride; 6) 5.0 mg *l*-Methadone Hydrochloride; 7) 5.0 mg *d*-Methadone Hydrochloride; 8) 5.0 mg *l*-Methadone Hydrochloride and 5.0 mg *d*-Methadone Hydrochloride; 9) 5.0 mg *l*-Methadone Hydrochloride and 3.2 mg dextrorphan tartrate; 10) 1.6 mg levorphan tartrate and 2.5 mg *l*-Methadone Hydrochloride; 11) saline placebo; 12) 60 ml 95% ethyl alcohol; 13) 60 ml 95% ethyl alcohol and 3.2 mg dextrorphan tartrate; 14) 60 ml 95% ethyl alcohol and 5.0 mg *d*-Methadone Hydrochloride. The ethyl alcohol was always given orally to the fasting subjects. Drug treatments were randomized in 2 groups, namely, 1-11 and 12-14, and neither the operator nor the subjects knew what drug combinations were employed.

Results. The results of this investigation are summarized in Table I. If visual inspection was insufficient to determine the significance of desired comparisons between drug treatments, the Null Hypothesis and Student's "t" Test(9) were applied. The statistical analysis is summarized in Table II.

A comparison of treatments 1 and 2 indicates that on this type of experimental pain, 3.2 mg levorphan tartrate possesses about one and a half times the analgetic potency of 2.0 mg. A comparison of treatments 2 and 6 indicates that the doses of the *l*-isomers,

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* The levo form of 3-hydroxy-N-methylmorphinan (generic designation levorphan) and its dextro form (generic designation dextrorphan) used as tartrates and supplied by Hoffmann-LaRoche, Inc., Nutley, N. J.

† The levo and dextro forms of 6-dimethylamino-4,4-diphenyl-3-heptanone were made available by Eli Lilly and Co., Indianapolis, Ind., as *d*- and *l*-Methadone Hydrochlorides.

‡ This investigation was supported by a grant from Hoffmann-LaRoche, Inc., Nutley, N. J.

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TABLE I. Summary of Data.

mg Treatment	(1) 2.0 levorphan tartrate	(2) 3.2 ” ”	(3) 3.2 dextrorphan ”	(4) 3.2 levorphan ” & 3.2 dextrorphan ”	(5) 3.2 levorphan ” & 5.0 *d*-Methadone hydrochloride	(6) 5.0 *l*-Methadone hydrochloride	(7) 5.0 *d* ” ”	(8) 5.0 *l* ” ” & 5.0 *d* ” ”	(9) 5.0 *l* ” & 3.2 dextrorphan tartrate	(10) 1.6 levorphan tartrate & 2.5 *l*-Methadone hydrochloride	(11) saline placebo	(12) 60 ml 95% ethyl alcohol (orally)	(13) 60 ml *idem.* & 3.2 mg dextrorphan tartrate	(14) 60 ml *idem.* & 5.0 mg *d*-Methadone hydrochloride
x	7.4	11.5	.7	5.0	5.1	13.6	1.7	5.1	4.2	11.6	.7	8.3	3.2	2.4
n	10	13	6	6	5	6	6	6	6	6	7	6	6	6
v_x	1.08	.34	.52	.01	.04	1.16	1.11	.02	.73	1.39	.40	1.15	1.05	1.15
s_x	1.04	.58	.72	.1	.2	1.08	1.05	.14	.85	1.18	.63	1.07	1.03	1.07

x = Mean % increase in pain threshold produced by drugs and drug combinations.
n = No. of observations. v_x = variance of mean. s_x = Stand. dev.

levorphan tartrate (3.2 mg) and *l*-Methadone Hydrochloride (5.0 mg), possess equal analgetic potency.

A comparison of treatments 2, 3, and 4 and of 6, 7, and 8, respectively, shows that the analgetic activity of 3-hydroxy-N-methylmorphinan and of 6-dimethylamino-4,4-diphenyl-3-heptanone resides in the levo isomers of these compounds and that the analgetically inactive dextro isomers are able to inhibit the analgetic activity of their stereo-isomers. Comparison of treatments 2, 4, and 5 and of 6, 8, and 9, respectively, indicates that the analgetic activity of levorphan tartrate and *l*-Methadone Hydrochloride is inhibited by the inactive dextro isomer of the other compound to the same degree as by its own inactive stereo-isomer. A comparison of treatments 2, 6, and 10 shows that the effects of a combination of half the dose of each levo isomer are additive in nature.

The inhibition of analgesia of levorphan tartrate and of Methadone Hydrochloride by the dextro isomers of either compound and the additive effect of a combination of the levo isomers suggest that the mechanism of action of these agents is the same. It would be plausible to assume that the inhibition of morphine analgesia effected by dextrorphan tartrate(4) would also be effected by *d*-Methadone Hydrochloride.

It was of interest to determine the effects of dextrorphan tartrate and *d*-Methadone Hydrochloride on the analgesia produced by ethyl alcohol. A comparison of treatments 12, 13, and 14 indicates that both compounds were equally effective in inhibiting the analgesia produced by ethyl alcohol. This inhibition suggests that some aspect of alcohol analgesia involves a mechanism similar to that of the morphine-type analgetics or that some aspect of the mechanism of alcohol analgesia may be mediated through their site of action.

Nausea and dizziness were reported occasionally by 5 subjects, with emesis following in 3 after they had received 3.2 mg levorphan tartrate. The same 3 subjects reported nausea and dizziness after they had been given 5.0 mg *l*-Methadone Hydrochloride with emesis following in one. No side effects were reported after the administration of 2.0 mg levorphan tartrate. Emesis was produced in one subject by the combination of 1.6 mg levorphan tartrate and 2.5 mg *l*-Methadone Hydrochloride. In the preliminary training period one individual—a 7th subject—was eliminated since he showed a drug sensitivity characterized by an extensive rash to levor-

TABLE II. Statistical Analysis.

Between treatments	1 vs 2	2 vs 6	2 vs 10	3 vs 4	3 vs 5	3 vs 7	4 vs 5
d	4.1	2.1	.1	4.3	4.4	1.0	.1
V_d	1.42	1.50	1.73	.53	.56	1.63	.05
s_d	1.19	1.23	1.32	.73	.75	1.28	.22
"t"	3.45	1.71	0.008	5.90	5.87	.78	.46
N	21	17	17	10	9	10	9
P	.01–.001	.30–.20	>.90	<.001	<.001	.50–.30	.70–.50

Between treatments	6 vs 10	7 vs 8	7 vs 9	7 vs 11	8 vs 9	9 vs 11	2, 6, and 10 vs 4, 5, 8, and 9
d	2.0	3.4	2.5	1.0	.90	3.5	7.2
V_d	2.55	1.13	1.84	1.51	.75	1.13	.77
s_d	1.60	1.06	1.36	1.23	.87	1.06	.88
"t"	1.25	3.21	1.84	.77	1.03	3.30	8.18
N	10	10	10	11	10	11	46
P	.30–.20	.01–.001	.10–.05	.50–.30	.30–.20	.01–.001	<.001

Between treatments	11 vs 13	11 vs 14	12 vs 13	12 vs 14	13 vs 14	11 vs 13 and 14	12 vs 13 and 14
d	2.5	1.7	5.1	5.9	.8	2.1	5.5
V_d	1.45	1.55	2.20	2.30	2.20	.92	1.67
s_d	1.21	1.25	1.48	1.52	1.48	.96	1.29
"t"	2.07	1.36	3.44	3.81	.54	2.19	4.26
N	11	11	10	10	10	17	16
P	.10–.05	.30–.20	.01–.001	.01–.001	.70–.50	.05–.02	<.001

d = Difference between means. V_d = Variance. s_d = Stand. dev. N = No. of degrees of freedom. P = Probability.

phan tartrate, as proven by a positive response to the intracutaneous injection of 0.16 mg levorphan tartrate; this sensitivity did not extend to *l*-Methadone Hydrochloride.

Summary and conclusions. 1. The relative analgetic potencies of the tartrates of the optical isomers of 3-hydroxy-N-methylmorphinan and of the hydrochlorides of Methadone were determined in normal human subjects on finger nail bed pain. 2. An inhibitory effect of the dextro isomers on the analgetic activity of the levo isomers and an additive effect of a combination of the levo isomers were demonstrated. 3. An inhibitory effect of the dextro isomers on ethyl alcohol analgesia was observed. 4. The results of this investigation suggest that the mechanism of action of the morphine-type analgetics studied is the same.

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Resistance to Reinfection in Experimental Brucellosis. (20703)

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Elberg and Silverman's report(1) contains an elaborate discussion and bibliography on immunology of brucellosis up to 1950. Few publications have appeared during the last 3 years, while this work was being done(2,3). Information in the literature, about the effect of the presence or absence in tissues of the brucella of primary inoculation on the duration of resistance to reinfection in experimental animals, is meager. The purpose of this work was to study this aspect of the problem.

Materials and methods. Following McEwen's technic(4), 2 smooth strains of *Br. abortus* were used: Strain No. 1489, a Co_2 dependent organism, and strain "Sablin" which grew aerobically and under partial CO_2 tension. The anaerobic culture was used for primary inoculations; reinoculations were done with the aerobic organisms. Animals were inoculated subcutaneously or/and intracardially with .1 ml of a saline suspension matching $BaSO_4$ standard No. 1, prepared with a 48-hour-old growth on tryptose agar. Preliminary experiments showed that this amount produced infection consistently in all animals, when given subcutaneously or intracardially, as evidenced by abundant growths from spleen and/or lymph nodes one month after inoculation.* Castañeda's method(5) was used routinely for blood cultures and organs were cultured by streaking the cut surface of tissues or macerated material on tryptose agar. The number of organisms in tissues was estimated by depositing measured amounts of diluted macerated tissue on the surface of agar plates. The history of the strains and description of methods are given in detail in previous publications(6,7).

In preliminary experiments cultures were made of the bile, urine and all tissues of guinea pigs infected subcutaneously with the 2 strains of *Br. abortus* utilized in this study. Organisms were recovered most frequently from the spleen, pooled lymph nodes, bone marrow and lungs. Infection lasted longest in spleen and lymph nodes. Brucellae were recovered from these tissues in all animals 3 months after inoculation but were isolated only occasionally after 6 months. Brucellae have been recovered only once from the urine and twice from bile in approximately 100 animals examined from one week to 6 months after infection. The acriflavine test, applied routinely to a large number of colonies recovered from the tissues, was consistently negative.

Results. The data presented in the following experiments are typical of the results ob-

TABLE I. Resistance to Reinfection among Guinea Pigs Experimentally Infected with *Br. abortus*.

Days after infection‡ when animals were reinoculated	No. of animals reinoculated	Cultivation of Brucellae from spleen and/or lymph nodes	
		Organism of original infection	Organism of reinoculation
11	6	All,* 4+†	5 — 1 2+
30	6	" 4+	All, —
58	6	" 3+	" —
94	1	3+	" —
	1	1+	
	1	—	
128	2	—	" —
	1	1+	
170	2*	4+	—
	1	—	1+
	2	—	—
225	2*	—	—
	1	—	—
	1	2+	—
	1	—	1+
420	1*	—	—
	2*	—	4+

Controls not included; all positive.

* Animals killed 2 wk after reinfection was attempted; other animals killed 4 wk after reinoculation.

† 1+ = 1 to 5 colonies, 2+ = 6 to 15 colonies, 3+ = 16 to 30 colonies, 4+ = 30 to 75 colonies, 5+ = more than 75 colonies.

‡ All animals gave a positive blood culture at one time or another during exp.

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* The disadvantages of using a 100% endpoint were eliminated by determining in each case, active primary infection by blood cultures.

TABLE II. Resistance of Guinea Pigs, Experimentally Infected with *Br. abortus,* to Intracardial and Subcutaneous Reinoculation.*

Days after reinoculation when animals sacrificed	Route of reinoculation	No. of animals: Normal control	No. of animals: Reinoculated†	Cultures from spleen and/or lymph nodes: Brucella of original infection	Cultures from spleen and/or lymph nodes: Brucella of reinoculation
3	S	2			3+
			2	—	—
			1	C	1+
			1	1+	1+
	I	2			5+
			1	1+	—
			1	—	—
			2	1+	1+
10	S	2			5+
			2	—	—
			1	1+	—
			1	—	2+
	I	2			5+
			1	1+	—
			1	—	1+
			1	—	—
40	S	2			5+
			2	—	—
			1	1+	—
	I	2			5+
			1	1+	—
			2	—	—
8	I	3			5+
			2‡	—	—
			2§	4+	—

* 5 mo. after primary infection. † 3 animals died and discarded. ‡ Reinoculated 7 mo. after infection. § Reinoculated 2 mo. after infection.

S = Subcutaneous, I = Intracardial, C = Contaminated.

tained repeatedly with relatively small groups of animals.

Exp. 1 was done to study any correlation between resistance to reinfection and the presence in the tissues of the organisms of primary inoculation. Forty-eight guinea pigs were injected subcutaneously and subsequently challenged, at different times, by the same route. Animals were sacrificed and tissues were cultured 2 or 4 weeks after reinoculation. Eleven guinea pigs died of intercurrent infection and were discarded. Results are shown in Table I. From 3 to 6 normal controls, not included in the table, were always injected at the time of reinoculation and all gave abundant growths from spleen and lymph nodes. Cultivable brucellae disappeared from the tissues in some animals earlier than in others. The bacteria of primary inoculation were recovered in one instance 225 days after infection; this animal was resistant to reinoculation. Two animals challenged 170 days and 225 days respectively after primary inoculation, from which organisms of the original infection were not cultured, were susceptible to reinfection. Three animals, reinoculated 420 days after infection gave negative cultures for brucellae of the original infection. One of these animals was resistant and 2 were susceptible to reinoculation. The organisms of reinoculation were never recovered from the tissues in the presence of the brucellae of primary infection, except in one instance. The exception was a guinea pig reinoculated 11 days after infection.

Exp. 2. Failure to recover the organisms of subcutaneous reinoculation from the spleen and lymph nodes of resistant animals, at a time when abundant growth was obtained from the tissues of controls, may be due to 1) inability of organisms to penetrate into the deep tissues 2) rapid disposal of the bacteria after reaching the internal organs. The purpose of this experiment was to investigate these possibilities. Twenty-four animals were divided into 2 groups of 12 each, 5 months after primary subcutaneous inoculation. One group was reinoculated subcutaneously and the other intracardially. Animals from each group were sacrificed 3, 10 and 40 days after reinoculation. Results are shown in Table II. The results obtained in a separate experiment with 4 animals challenged intracardially 2 or 7 months after infection, and sacrificed 8 days after reinoculation, are also included in this table. Brucellae penetrated the internal organs when injected subcutaneously into animals 5 months after primary infection, but they disappeared rapidly. Similarly, organisms injected intracardially also cleared promptly from the tissues in resistant animals. Infected guinea pigs reinoculated subcutaneously exhibited the local reaction described by Burnet(8) characterized by small abscesses which in many instances ruptured to the outside and eventually healed. In normal controls this reaction was less frequent and milder. The pus always gave abundant growth of brucellae. This local reaction was not a major contributing factor in resistance to reinfection among previously infected animals challenged subcutaneously. Cultures from spleen and/or pooled lymph nodes have been reported here as the sole criterion of infection because when cultures from these tissues were negative those of the blood, bone marrow, liver and other organs were also negative. In many cases streaked cultures of the cut surface or organs and macerated tissue, as well as a quantitative estimation of the number of organisms, were done in the same animal. Under these circumstances the number of animals sacrificed on any single day was necessarily small. These experiments, however, have been repeated using small groups of animals and the same results reported above have been obtained consistently.

Exp. 3 was attempted in order to meet criticism based on the assumption that intracellular brucellae cannot be cultured by the methods employed. Exp. 2 and 3 were repeated using, in addition to the culture technics utilized previously, cultivation of tissues after submitting them to rapid vibration in a Mickle tissue disintegrator. Preliminary experiments showed that disintegration of 10 ml of tissue suspensions, at a given amplitude, for 5 minutes gave best results. Estimation of the number of organisms in tissue suspensions (lymph nodes, spleen, and liver) before and after disintegration showed no significant differences. The results obtained with disintegrated materials were essentially the same as with the other methods and the findings reported above were fully corroborated. When disintegrated citrated blood was deposited directly on the surface of agar media growth appeared earlier than in Castañeda's double medium(5). On solid media, inoculated with same amount of undisintegrated and disintegrated blood, growth was more abundant and colonies were usually larger after disintegration. This observation, made in resistant animals and in those in which agglutinins had not yet developed, suggests that some bacteria may have been dislodged from the phagocytes, making the nutrients in the medium more readily available.

Summary and conclusions. 1. The purpose of the present work was to determine if a relationship exists between resistance to superimposed infection and the presence of the organisms of primary inoculation in the body of guinea pigs infected with *Br. abortus*. The organisms of reinoculation never persisted in the tissues in the presence of the brucellae of primary infection, except in one instance. The exception was a guinea pig reinoculated 11 days after infection. Some animals, from which the organisms of the original infection were not recovered from any of the tissues, were resistant to reinfection. Resistance waned gradually after the organisms of primary inoculation could no longer be recovered from the tissues. 2. A local reaction with abscess formation was produced at the site of subcutaneous inoculation in infected guinea pigs; brucellae were recovered in large numbers from these lesions. The organisms of reinfection penetrated into the deep tissues but disappeared promptly. This suggests that the local reaction was not a major contributing factor in resistance to reinfection. When previously infected animals were injected intracardially the brucellae of reinfection also disappeared rapidly from the tissues. 3. In order to meet the criticism that intracellular brucellae could not be cultivated by routine methods, all tissues were cultivated after disintegration by rapid vibration, under optimal conditions. The results obtained with this technic corroborated the findings obtained with other methods. 4. It is puzzling to see that the *Brucella* of original infection can persist for months in the tissues from which the organisms of reinoculation disappear rapidly. An explanation for this phenomenon is not apparent and these studies are being extended in an attempt to clarify this point. 5. Cultivation of disintegrated blood on the surface of solid media constituted an improvement over other methods used routinely for the isolation of brucellae from the blood.

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Relative Biological Effectiveness of Fast Neutrons, Gamma Rays, X-Rays on Grasshopper Nymph Ovarioles. (*Melanoplus differentialis*) (20704)

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In previous communications(1-3) it was demonstrated that anabolic processes are more susceptible to ionizing radiations than catabolic processes. In order to examine this phenomenon further a tissue was required in which growth, in the sense of the synthesis of cellular material, takes place, in the absence of cell division. These requirements are fulfilled by the nymphal grasshopper ovariole and its developing eggs. During early oogenesis in the ovarioles, the newly differentiated unfertilized egg is approximately 35 μ in diameter, but within 45 days this cell attains a size of 4 cu mm, increasing in volume by a factor of 1.6×10^5. Throughout this period (still before fertilization) the egg contains the diploid number of chromosomes. Meiotic divisions begin just prior to laying and are completed after the eggs are laid, as previously shown by Slifer and King(4).

In accordance with expectation the developing eggs were found to be highly radiosensitive: Whereas a total-body dose of 1500 r of 200 kv x-rays was necessary to kill 50% of the grasshopper nymphs in a 10-day period, only 350 r or its equivalent was sufficient to destroy completely most of the eggs in the ovarioles. In this paper the relative biological effectiveness of Co^{60} gamma rays and fast neutrons is compared with 200 kv x-rays for the production of this irradiation effect.

Material and methods. Fifth or sixth instar grasshopper nymphs were exposed to whole body x-radiation in doses ranging from 52 to 801 r, from a 200 kv, 15 ma, oil-cooled x-ray machine; HVL: 0.25 mm Cu; no additional filtration. The grasshoppers were exposed in a covered Petri dish made of cellulose acetate. In each dosage series 10 or 12 grasshoppers were kept as controls and an equal number were irradiated. Gamma and fast neutron exposures were all made in the gamma-neutron radiation chamber recently described by Vogel *et al.*(5). Slow neutrons from the thermal column of the Argonne CP_3' Reactor were allowed to impinge upon a sheet of uranium in order to produce fast neutrons. The fission spectrum was modified by 3 inches of lead around the uranium. Pure gamma radiation was obtained from 18 sources of cobalt 60 located at the opposite end of the chamber from the uranium. The grasshoppers were exposed in lucite cages usually used for exposure of small mammals. These cages had been standardized as to isodose groups for both gamma and neutron irradiations. Exposures were carried out at a dose rate of approximately 13 r/min for gamma rays and 2-4 rep/min for fast neutrons. During irradiations the animals were kept at approximately 75°F and 50% relative humidity with the aid of an Aminco climatizer which circulated air to both experimentals and non-irradiated controls. Sixteen days following each irradiation the ovaries were removed and the exposed ovarioles were observed under a dis-

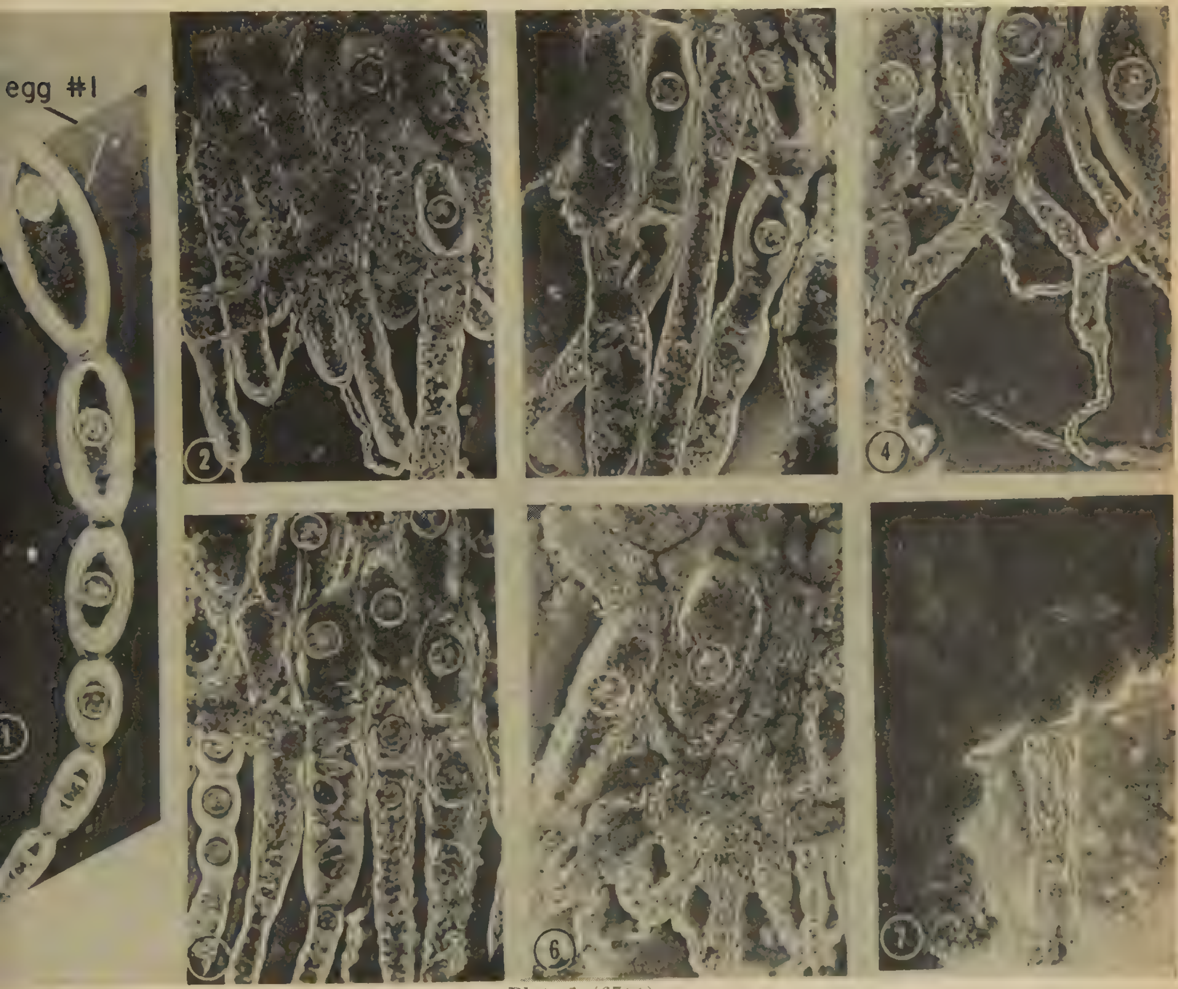


Plate 1 (67×)

FIG. 1. Normal control ovariole showing a strand of ova with egg #1 labelled.

FIG. 2. Grasshopper ovary that received total-body radiation of 350 r x-rays 16 days before it was photographed.

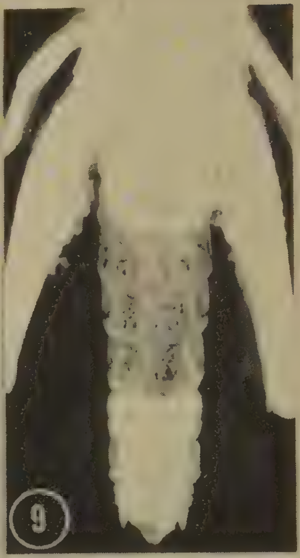
FIG. 3. A number of ovarioles 16 days after 407 r Co^{60} gamma rays.

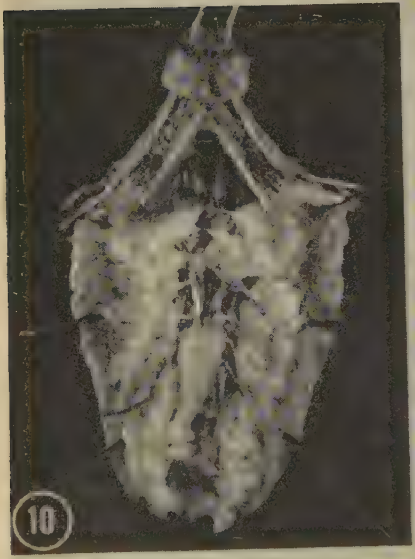
FIG. 4. Ovarioles exposed to 480 r x-irradiation.

FIG. 5. Ovarioles 16 days after fast neutrons bombardment with 14.1 rep.

FIG. 6. 22.5 " .

FIG. 7. 101.4 " .





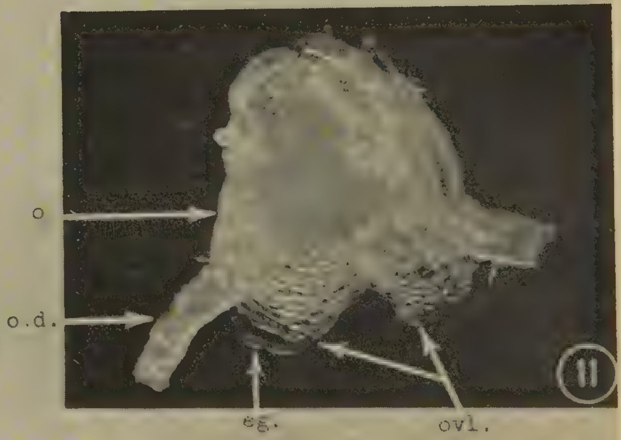


Plate 2

FIG. 8. Irradiated grasshopper nymph, fifth instar, 3×.

FIG. 9. Grasshopper showing abdominal region below which the ovary is found (shaded area indicates location of ovary), 6×.

FIG. 10. Dissected grasshopper nymph with intact ovary, 3×.

FIG. 11. Ovary dissected out (normal), 15×.

O—ovary Od—oviduct Eg—egg Ovl—ovarioles

TABLE I.
(Irradiation data.)
X-rays (200 kv 15 ma); 10 animals in each experiment.*

Date of exposure	Dose rate (r/min.)	Exposure (min.)	Total dose (r)	Irradiation effect†	
2/ 4/52	40.2	1.3	52	3—‡	Normal or control ovary (see Fig. 1 and 11).
"	"	2.5	101	"	
3/ 7	34.4	6.6	227	2—	No evident injury to eggs; ovary retarded in growth.
"	"	8.8	303	"	
"	"	10.2	351	+	Destruction of every egg in each ovariole, except egg #1, the most mature (as in Fig. 2, 4, and 6).
2/ 4	80.1	5	401	"	
"	"	6	481	"	
"	"	8	641	2+	Destruction of every egg in each ovariole.
"	"	10	801	3+	Complete destruction of each ovary (as in Fig. 7).
				Co^{60} gamma rays:	
2/19/53	12.9	16.26	210	—	Some destruction of eggs; ovary retarded in growth.
3/10	12.8	25.4	325	"	
"	"	30.0	384	"	
"	"	31.8	407	±	Destruction of several eggs in most ovarioles, but more than one egg evident in most ovarioles of each ovary (as in Fig. 5).
2/19	12.9	32.5	420	+	
				Fast neutrons:	
	(rep/min.)		(rep)		
7/28/52	2.5	4.	10	—	
2/10/53	4.61	3.06	14.1	±	
7/28/52	2.5	9.	22.5	+	
2/10/53	4.61	5.5	25.3	+	
7/28/52	2.5	17.	42.5	2+	
2/10/53	4.61	11.	50.7	2+	
6/30/52	1.96	41.	80.4	3+	
"	2.21	41.	90.6	"	
2/10/53	4.61	22.	101.4	"	
6/30/53	2.65	41.	108.7	"	
				Control animals:	
10 controls for each of 7 irradiation dates—(2 controls for 3/7/52 died, but ovaries appeared normal).					

* *i.e.,* number of grasshoppers whose ovaries were examined after irradiation. There were some deaths among irradiated animals, especially at higher neutron doses. For example, 11 grasshoppers out of 20 died after receiving 101 rep fast neutrons (LD$_{55/16}$ days).

† Criteria used to judge biological effects on grasshopper ovary 16 days after irradiation.

‡ With x-ray doses below 200 r, the egg primordia divide mitotically and fill the ovarioles with embryonic cells which are incapable of differentiating into eggs.

secting microscope at 40×. Cytological studies on the living eggs were carried out with the aid of the Leitz Ortholux phase contrast system.

Dosimetry. During the actual irradiation periods, Victoreen thimble chambers were inserted into standard positions in the dosimetry columns of the lucite animal cage(5). One of these chambers has been calibrated in terms of roentgens-equivalent-physical (rep) when exposed to the fast neutron field. Dr. H. H. Rossi of Columbia University used a tissue-equivalent chamber of 10.1% hydrogen composition for this calibration. Factors have been obtained to relate the response of other Victoreen chambers (25 r, 100 r, 250 r) to this calibrated chamber. Thus, after corrections for temperature and barometric pressure, the response of these chambers can be corrected to give a dosage measurement in standard units. One of the Victoreen 25 r thimble chambers with a 4 mm plastic cap was also calibrated by the National Bureau of Standards for gamma rays of Co^{60} in terms of International Roentgens. Whenever fast neutrons are used there is a problem of contamination with gamma rays. By the use of films and a carbon chamber(6) it was concluded that the gamma ray contribution to the tissue dose in the neutron field was approximately 7% of the rep of fast neutrons. Slow neutrons were effectively eliminated by appropriate shields of boron carbide.

Results. As a biological criterion for the evaluation of irradiation damage by the various ionizing radiations we have taken the destruction of every egg in the 80 or more ovarioles of the grasshopper ovary with the exception of egg No. 1, the most advanced ovum, found adjacent to the oviduct (Fig. 1 and 11).

The experimental data are presented in Table I.

In order to produce similar damage to the ovarioles, the following minimum doses were required: 350 r of x-rays; 420 r of gamma rays; 22.5 rep fast neutrons. Thus, assuming gamma radiation to be 1, the relative biological effectiveness (R.B.E.) of these radiations for this result was:

$$\text{X-rays} = \frac{420}{350} = 1.2$$

$$\text{Fast neutrons} = \frac{420}{22.5} = \sim 19$$

Discussion. The state of an irradiated tissue with respect to age, metabolism, and degree of differentiation is important in considering its relative radio-sensitivity. The high anabolic rate in the formation of the grasshopper egg within the ovariole may well be a major cause for its sensitivity to ionizing radiations. It was found that the egg primordia, after low x-ray doses, were incapable of differentiation. The cells divided mitotically, but, instead of developing into eggs, they filled the ovarioles with small, undifferentiated, embryonic cells. This is in agreement with recent observations in embryonic tissue where differentiation is more susceptible to irradiation than anabolism itself(3). Invertebrates are ordinarily considered more radio-resistant than vertebrates for whole-body radiation. This apparent resistance may be due in part to metabolic rate and to the type of transport system involved. In the grasshopper two paths exist for energy transfer to any given cell: the tracheal system, supplying oxygen (directly into the cells), eliminating carbon dioxide and water, and the so-called "blood" or coelomic fluid which supplies organic foods and eliminates nitrogenous wastes from each cell through the malpighian system.

The results of these experiments indicate that 420 r of Co^{60} gamma rays are equivalent to 350 r of 200 kv x-rays and to 22.5 rep of fast neutrons for the criterion selected to measure the degree of destruction of the grasshopper ova. Gray(7) and Ehrenberg and Nybom(8) have published the following mean linear ion densities for these radiations:

	Ion pairs/μ
Co^{60} gamma rays	8
200 kv x-rays	80
Fast neutrons (Stockholm cyclotron, 1-11.5 Mev)	700

(The mean energy of the fast neutrons used in our experiments was approximately 1 Mev with the highest flux at about 0.7. The linear ion density is therefore, according to the above data, greater than 700 ion pairs/μ.)

It is apparent from these figures that the R.B.E. of these 3 ionizing radiations, for this particular effect, is in the same order as their respective ion densities, gamma radiation requiring the highest dose and neutrons the lowest. Zirkle(9) has emphasized the importance of specific ionization and linear energy transfer in many radiobiological effects. The R.B.E. of gamma rays to fast neutrons for this biological effect (18-19) is the highest yet observed in experiments run in our gamma-neutron chamber. For example, the R.B.E. for acute 30-day lethality in the female mouse (CF-1) after 90-minute exposures to these same ionizing radiations is 4.4 ± 0.05(10).

Although there is a 16-day interval necessary between irradiation and evaluation, it is hoped that the grasshopper ovary may serve as a useful biological dosimeter. The insects are easily maintained in the laboratory, and the removal and examination of their ovaries can be done successfully with some experience. Our results, listed in Table I, show that doses of fast neutrons in excess of 80 rep completely destroy each ovary, but, for doses in the range 10-50 rep, we believe that our criteria can be used by other investigators to give an approximation of fast neutron doses.

It is clear from the data that the neutron dosages are less accurate than the other radiations for the criterion effect, since the separation of experimental dosages is higher in the effective range. In an attempt to test the accuracy and reproducibility of the criteria used, 5 groups of grasshoppers were recently irradiated with 14, 16, 18, 20, and 22 rep respectively, and their ovaries were later examined by an investigator who did not know the doses given to the insects. The 22 rep group was diagnosed in the single plus category and was named correctly as the group receiving the highest dose. This test substantiated the fact that 22 rep of fast neutrons was the minimum dose for the plus criterion. The grasshoppers that had been exposed to 14 rep were estimated to have received "about 15 rep" and these were placed in the ± category from a study of their ovarioles. However, there was enough variation in the ovaries of the remaining three groups so that they could not be clearly distinguished from each other and could only be termed "intermediate between 14 and 22 rep."

It is realized that this dosimeter gives at present only an approximation of the dose of fast neutrons within the rather narrow range of 10-50 rep. However, there is no reason to believe that such dosimeters cannot be improved both in quantitative accuracy and in reproducibility. Although biological materials will always possess variability, it is possible that ionizing radiations may be measured by several carefully selected biological dosimeters, each highly sensitive to a specific dose range of the radiation involved.

Summary. In order to destroy every egg in each grasshopper ovariole, with the exception of the most advanced, it was necessary to irradiate the total body with 350 r (200 kv) x-rays, 420 r Co^{60} gamma rays, or 22.5 rep of fast neutrons. Thus, the relative biological effectiveness of Co^{60} gamma rays: 200 kv x-rays: fast neutrons, for this specific effect, is 1.0: 1.2: 19. This is correlated with relative specific ionization and linear energy transfer. It is suggested that damage to the grasshopper ovariole could be used as a biological dosimeter for fast neutron doses in the range 10-50 rep.

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Tryptophan Metabolism in Rice Moth Larva (Corcyra Cephalonica St.)
(20705)

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Interest in the metabolism of tryptophan in the rice moth larva arose from the observation made by Sarma(1) that the larvae maintained on a tryptophan-rich, pyridoxine-deficient diet excreted yellow-colored feces. This revealed a striking parallel between mammals and insects, as regards tryptophan metabolism, since rats, rabbits, dogs and other mammals under similar conditions had been known to excrete the pigment metabolites, kynurenine, 3-hydroxykynurenine, xanthurenic acid etc.(2-5). Xanthurenic acid, the most characteristic metabolite in pyridoxine deficiency in mammals, was shown to be absent in the larval excretory pigment, as the latter gave a negative test with iron salts. Earlier work on tryptophan metabolism in insects has been mainly in connection with its function as a precursor of the pigments, ommochromes, found in the eyes and tissues. Here tryptophan undergoes conversion via kynurenine and hydroxykynurenine to the complex pigment molecule(6-8).

Paper partition chromatography has in recent years greatly facilitated the investigation of tryptophan metabolism in mammals. In the present paper, the larval excretory pigment has been analysed by the technic of circular paper chromatography first developed by Rutter(9) and later adapted to the resolution of amino acids in biological materials by Giri(10). In a preliminary communication Sundaram and Sarma(11) have reported the identification of kynurenine and hydroxykynurenine in the larval feces.

Methods. Whatman No. 1 filter circles, 27 cm in diameter, were used and the method employing the detachable wick described by Giri(10) was followed. Two solvent mixtures were tried, namely, butanol-acetic acid-water (4:1:5)(4) and butanol-methanol-benzene-water (1:2:1:1)(12). The former gave by far the better resolution in the form of compact spots and was used throughout the experiments reported herein. The effective distance traversed was about 10 cm, the time taken varying from 3 to 5 hours. Ultraviolet fluorescence and Ehrlich's reagent(13) were employed for the detection of the metabolites on the chromatogram. Quinolinic acid was located as the yellow chelate compound it forms with ferrous ion. In addition to being chromatographed individually, the metabolites were resolved in various combinations. The experimental technic for the study of tryptophan metabolism in the rice moth larva has been described(1,11). Rice moth larvae were kept on the appropriate experimental diet till the major portion of the diet had been consumed (1-1½ months). The feces excreted by pyridoxine-deficient and pyridoxine-fed larvae on diets supplemented with DL-tryptophan at a level of 1% were collected separately from the residuary diet and thoroughly extracted with distilled water. The extracts were concentrated *in vacuo* at 55°, made 60-70% alcoholic by addition of rectified spirit, centrifuged and the clear supernatant used for paper chromatography. Pure tryptophan metabolites were run alongside for purposes of comparison and identification of the constituents of the larval excretory pigment. After irrigation with the solvent mixture, the chromatograms were developed as mentioned above.

Results. The R_f values of the several tryptophan metabolites with details of identification tests employed are presented in Table I and compared with the values obtained by us according to the method(14) of ascending paper chromatography. In general, it is found that the R_f values obtained by circular paper chromatography are slightly higher. Formylkynurenine gave a second spot (R_f on circle .91) much paler than the main one. Kynurenic acid and 3-hydroxyanthranilic acid left green streaks behind the main spots.

The results of analysis of the extracts of

TABLE I. R_f Values of Tryptophan Metabolites.

Metabolite	Ultraviolet fluorescence	Ehrlich's reaction	R_f value	
			Circular	Ascending
L-tryptophan	Negligible	Orange-yellow (violet*)	.73	.58
DL-kynurenine	Sky-blue	Yellow-orange (fades*)	.69	.57
Formylkynurenine	”	”	.70	.56
3-hydroxykynurenine	Green	Pink (fades*)	.64	.49
3-hydroxyanthranilic acid	Purple-blue	Light orange-yellow	.95	.90
Xanthurenic ”	Violet-blue	Nil	.78	.69
Kynurenic ”	Green	”	.74	.64
Quinolinic ”†	Nil	”	.55	.50
Anthranilic ”	Violet	Orange-yellow	.97	.95

* On exposure to HCl fumes.

† Yellow with ferrous sulphate.

R_f values given represent mean of values obtained in several runs, deviations being within ±.03.

TABLE II. Metabolites Excreted by Rice Moth Larvae.

Spot	R_f	Ultraviolet fluorescence	Ehrlich's reaction	Identity
			Pyridoxine-deficient (L–)	
1	.44	Grey	Nil	†
2	.52	”	”	†
3	.66	Green	Pink (fades*)	3-hydroxykynurenine
4	.71	Sky-blue	Yellow-orange (fades*)	Kynurenine
5	.74	Nil	Orange-yellow (violet*)	Tryptophan
			Pyridoxine-fed (L+)	
1	.67	Violet	Yellow	†
2	.72	Green	Orange-yellow (fades and turns violet*)	Tryptophan and an unidentified metabolite
3	.82	Blue	Nil	†
4	.85	Green	”	†

* On exposure to HCl fumes.

† Not identified.

R_f values given represent mean of values obtained in several runs, deviations being within ±.03.

the larval excreta are summarised in Table II and Fig. 1.

The influence of pyridoxine on tryptophan metabolism, indicated in a qualitative way by the absence of the yellow color in the feces of pyridoxine-fed larvae on a tryptophan-supplemented diet, was further elucidated by chromatography of the extracts L+ and L— (Fig. 1) side by side. The 4 spots given by the L+ extract (Table II) do not appear to correspond to any of the common tryptophan metabolites. They may represent either tryptophan metabolites hitherto unknown or normal excretory products of the larva having their origin in substances other than tryptophan. It is significant, however, that these metabolites are absent in the L— extract. Similarly spots 1 and 2 given by the L— extract have not been identified so far. Similar unidentified metabolites have been reported by Dalgliesh(5) and by Reddi and Kodicek(13) in mammalian excretions and by Mason and Berg(12,15) in their tissue slice experiments on tryptophan metabolites and in the urine of rats dosed with D-tryptophan.

Kynurenine and 3-hydroxykynurenine, which are present in the L— extract but absent in the L+ extract (Table II and Fig. 1). constitute the yellow pigment excreted in pyridoxine deficiency. 3-hydroxykynurenine, identified in the excretion of mammals only very recently(4), has been found by us to be excreted by larvae fed tryptophan in the absence of pyridoxine. It was considered until 1951 as only a hypothetical intermediate in the tryptophan-niacin transformation in mammals in view of its demonstrated role in this series of reactions in *Neurospora*(16) and of

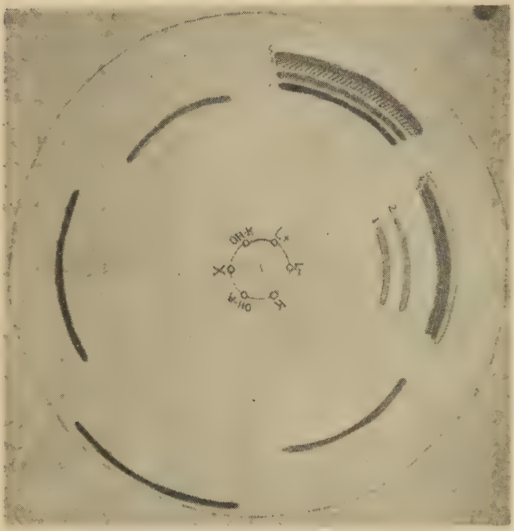

FIG. 1. Diagrammatic reproduction of circular chromatograms. K—Kynurenine; OH-A—3-Hydroxyanthranilic acid; X—Xanthurenic acid; OH-K—3-hydroxykynurenine; L+—Extract of feces of pyridoxine-fed larvae on tryptophan-supplemented diet; L– —Extract of feces of pyridoxine-deficient larvae on tryptophan-supplemented diet. (Vide Table II for details regarding spots given by extracts L+ and L–.)

its identification as the intermediary cn^+ substance formed from the v^+ substance, kynurenine, in the production of the insect pigments, ommochromes, from tryptophan(6-8). A tryptophan-niacin conversion scheme involving kynurenine and 3-hydroxykynurenine as intermediates and the role of pyridoxine therein are thus established also in the case of insects. The accumulation and the excretion of the kynurenines in pyridoxine deficiency by the rice moth larva are both in accord with the accepted mechanism of the metabolism of tryptophan to niacin in other organisms, where pyridoxal phosphate as coenzyme mediates the further conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid(4,5). The absence of xanthurenic acid in the larval excretory pigment already reported(1,11) is confirmed.

Summary. 1. The technique of circular paper chromatography has been adapted to the resolution of the various tryptophan metabolites. 2. Kynurenine and 3-hydroxykynurenine have been shown to constitute the yellow pigment excreted by rice moth larva on a tryptophan-rich pyridoxine-deficient diet. The absence of xanthurenic acid in the excretory pigment has also been demonstrated.

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Serum Potassium and Prolongation of Q-T Interval of Electrocardiogram in Acute Myocardial Infarction.* (20706)

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(Introduced by Henry Brody.)

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Recently, one of us (S.O.K.) has demonstrated that the Q-T interval of the electrocardiogram is definitely prolonged in acute myocardial infarction, especially during the early weeks of the disease(1,2). Several reports(3-5) have described prolongation of the Q-T interval, attributed to low serum potassium levels, in a variety of clinical conditions. It was the object of this study to determine whether or not the prolongation of the Q-T interval that does occur during the acute myocardial infarction can be correlated with a low serum concentration of potassium.

Method. Forty-seven consecutive cases of acute myocardial infarction admitted during the past year to the ward medical services of the Northern Division of the Albert Einstein Medical Center were studied. Sixty-seven potassium levels were obtained on blood specimens drawn on the same day as electrocardiograms were made on these patients. Serum potassium levels were performed using the flame photometer, and employing lithium as an internal standard. All determinations were done in duplicate. The normal serum potassium for this laboratory during the time of this study, based on random sampling of healthy blood donors, was 5.0 ± S.D. 0.46 meq/L. Measurement of the duration of the Q-T interval of the electrocardiogram done on the day of the serum potassium determination, and the correction to be made for cardiac rate, were performed as described in previous studies (1,2). The modified formula of Bazett was employed, $\text{Q-T}_c = \dfrac{\text{Q-T}}{\sqrt{\text{R—R}'}}$. The normal corrected Q-T interval was found to be 0.397 ± S.D. 0.015 second, with a range of 0.38 to 0.43 second. The serum potassium level will hereafter be referred to as [K+] and the corrected Q-T interval as Q-T_c.

TABLE I. Relation of Serum Potassium Concentration to Q-T_c in Myocardial Infarction.

No.	Q-T_c (sec.) Range	Avg	$[\text{K}^+]$ (meq/L) Range	Avg
44	.44–.50	.46 ± .02	4.1–6.3	5.3 ± .5
23	.36–.43	.40 ± .02	4.6–6.4	5.4 ± .43

Results (Table I). In 44 electrocardiograms the Q-T_c's were significantly longer than normal, ranging from 0.44 to 0.50 second (average 0.46 ± 0.02 second). The 44 serum samples drawn the same days as these electrocardiograms were done, revealed [K+]'s ranging from 4.1 to 6.3 meq/L (average 5.3 ± 0.5 meq/L).

In 23 electrocardiograms the Q-T_c's were normal or shorter than normal ranging from 0.36 to 0.43 second (average 0.40 ± 0.013 second). In this group 13 tracings were taken at a time when the patients were receiving digitalis. The 23 serum samples drawn on the same day revealed [K+]'s ranging from 4.6 to 6.4 meq/L (average 5.4 ± 0.43 meq/L). (Fig. 1)

Discussion. It has been demonstrated by this study that during myocardial infarction, at a time when the Q-T_c is definitely prolonged, the [K+] is within normal limits or even slightly higher than normal. It can therefore be concluded that the prolongation of the Q-T_c that does occur in acute myocardial infarction is not associated with a low level of serum potassium. Such prolongation, however, is noted in the presence of a normal or slightly higher than normal serum potassium concentration.

Why, then, is there a prolongation of the Q-T interval of the electrocardiogram during the early weeks in acute myocardial infarction? The factors of age, sex, species and body position seem to have little effect as previously dis-

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* Read by title annual session of Am. Heart Ass'n, Atlantic City, N. J., April 1953.

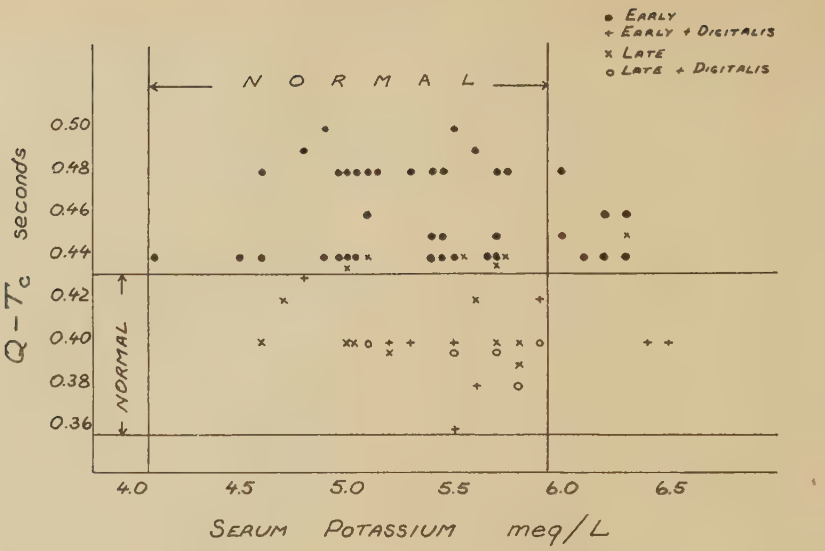

FIG. 1. Relation of serum potassium level to the Q-T_c early (first to fourth wk inclusive) and late (after fourth wk) in acute myocardial infarction.

cussed(2). Simple physical interference with electromotive forces has yet to be demonstrated adequately, but it seems hardly likely that it could be of a magnitude as to cause a delay of 0.04 to 0.08 second per cardiac cycle. Could the Q-T_c prolongation be associated with, or related to changes in the potassium concentration in heart muscle *per se,* rather than its concentration in the serum? Iseri and his co-workers(6) have recently demonstrated a marked lowering of heart muscle potassium in blocks taken from infarcted hearts. These investigators report a heart muscle concentration of 8.32 meq/100 g of wet tissue in normal hearts compared to 4.19 meq/100 g of wet tissue taken from hearts with acute infarction.

Lepeschkin and Surawicz(7) recently observed that "the true corrected Q-T duration is not increased and may even be shortened in typical hypopotassemia without hypocalcemia". In view of this report and our study, it appears entirely possible that the changes observed in the duration of the Q-T interval are entirely unrelated to the serum potassium concentration.

Summary. In a study of 67 electrocardiograms taken on 47 patients with acute myocardial infarction in conjunction with a similar number of serum potassium determinations, it was found that the [K^+] was either normal or slightly above normal at a time when the Q-T_c was prolonged, normal or shortened. The prolongation of the Q-T_c that occurs during acute myocardial infarction is not correlated with a low level of serum potassium. Other possibilities for prolonged Q-T_c are discussed.

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Effect of Intravenously Injected Radioisotopes on Metastatic Tumor Cells in Organs of the Mouse.* (20707)

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Several authors(1) have observed that inoculation of cell suspensions (brei) of apparently healthy organs from tumor-bearing mice induced occasionally tumor growth at the site of injection. However, these results were not used for systematic screening of viable tumor cells in various tissues partly because they were attributed by some investigators to "cancer virus" spread from the primary tumor, and mainly because injection of organ brei often provoked intense local reaction inhibiting growth of few tumor cells. We have found(1) that these difficulties can be overcome by using the intraperitoneal route for assay inoculation (graft). Moreover we have noted previously(2) that after introduction of a mixture of normal cells and tumor cells into the peritoneal cavity, only tumor cells grew as free cells in the peritoneal fluid, while normal tissue cells rapidly disintegrated. This method of using the peritoneal cavity as a location that selectively promotes growth of tumor cells has demonstrated consistently that for each tumor strain the frequency and the distribution in organs of metastatic cells followed a certain pattern(1).

It occurred to us that the growth of organized tumors from metastatic cells could be prevented by introduction into the organs of radioactive or chemical agents inhibiting, in well tolerated doses, the viability of malignant cells. We have shown previously(3-6) that direct contact with radioactive gold or iodine in sufficient concentration inhibited, in many instances completely, the growth of tumor cells. This effect was demonstrated by considerable decrease in their number per cmm of peritoneal or pleural fluid, by disappearance or abnormal appearance of their mitoses and by their inability to initiate growth after transfer into normal mice (biological test of viability). Moreover, it is known that colloidal Au^{198} is stored mainly in the liver and spleen(7), while the large insoluble particles of $CrPO_4$ suspension may be filtered from the blood by the capillary net in the lung(8). Therefore these radioisotopes were selected for trial of their effect on the viability of metastatic cells in the liver, the spleen and the lung.

Materials and methods. A. *Tumor and mouse strains.* Sarcoma strain S-37 was carried in CFW mice in serial intraperitoneal transfers as free cells growing in the peritoneal fluid(2). Technics of intraperitoneal and intrapleural inoculation of requisite numbers of tumor cells were described elsewhere(2). Ten to 50 millions of cells were inoculated in each series of experimental mice. B. *Treatment.* Radioactive material† was diluted with 0.85% NaCl solution to the concentration of 1 mc per ml. Doses of 0.3 to 0.35 mc (shown previously (4) to be well tolerated by mice) were injected by the i.v. or i.p. route. Diluted suspensions of chromic phosphate were shaken before each injection. C. *Pattern of the experiment.* Mice ("donors of organs") were inoculated with the same dose of tumor cells of the same strain and by the same route. In each series one group was left untreated (controls) and another group was treated on the day of inoculation (graft) or at various intervals before or after inoculation. Mice of both groups were sacrificed 4 or 5 days after inoculation. Their livers, spleens, kidneys and lungs were mashed separately, and the brei of each organ was injected intraperitoneally into normal mice. These mice ("recipients of organ brei") were examined after 10 days for the presence of tumor cells in their peritoneal fluid and for gross evidence of tumor growth in the peri-

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† Colloidal Au^{198} obtained through the courtesy of Dr. P. F. Hahn, Cancer Research Laboratories; chromic phosphate through the courtesy of Dr. D. L. Tabern, Abbott Laboratories, North Chicago, Ill.

TABLE I. Assay Organ Grafts (Assay for Presence of Tumor Cells in Organs) from CFW Mice Bearing Primary Intraperitoneal Sarcoma 37 Grafts and Treated with Radioisotopes.*

Series	Days between primary tumor graft and treatment	Organs examined			
		Liver	Spleen	Kidney	Lung
1†	5	8/2	6/4	9/1	8/2
	2	6/4	8/2	10/0	6/4
	0	5/5	5/5	8/2	6/4
	–2	7/3	6/4	8/2	8/2
	Controls	10/0	8/2	10/0	7/3
2	5	8/2	6/4	9/1	7/3
	2	1/9	3/7	10/0	4/6
	1	2/8	5/5	10/0	5/5
	0	1/9	4/6	9/1	4/6
	–2	1/9	4/6	10/0	4/6
	–5	10/0	5/5	10/0	7/3
	Controls	10/0	7/3	9/1	6/4
3	5	7/3	2/8	10/0	8/2
	2	2/8	1/9	8/2	9/1
	1	2/8	2/8	9/1	10/0
	0	1/9	1/9	10/0	8/2
	–2	2/8	2/8	8/2	9/1
	Controls	9/1	3/7	9/1	9/1
4	5	7/3	6/4	10/0	3/7
	2	6/4	4/6	9/1	1/9
	1	5/5	4/6	9/1	0/10
	0	6/4	3/7	10/0	0/10
	–2	5/5	4/6	10/0	1/9
	Controls	10/0	7/3	9/1	7/3

* For each group of 10 mice, results are presented as a fraction, the numerator indicating No. of takes and the denominator, No. of no takes.

† Primary tumor graft: 1st, 2nd and 4th series intraperitoneal, 3rd-intrapleural. Treatment with radiogold: 1st series intraperitoneal, 2nd and 3rd intravenous. Treatment with radioactive chromic phosphate—4th series intravenous.

Note: (1) second column, – before numbers indicates that treatment was given before primary tumor graft; 0 indicates simultaneous graft and treatment. (2) 3rd column, actually 15 to 20 mice were used in each group but for easier reading and interpretation of results they were calculated in table as per 10 mice. (3) Assay organ grafts were done 5 days after primary intraperitoneal grafts and the results recorded 10 days later.

toneal wall.

Results. The results are reviewed in Table I. The first series of experiments shows the results of the treatment with colloidal Au^{198} given at the site of inoculation in a closed cavity and thus directed in the first line locally against the primary growth and only to a lesser extent (since the absorption of colloidal material from the cavity in the blood is relatively low) against tumor cells disseminated into organs. Inverse experimental conditions were present in the second series of the table illustrating the effect of radioactive agents given intravenously and taken in large amounts from the blood by some organs, but only in very small amounts by the cavity containing primary growth. In the third series the growth was initiated in another cavity—the pleural cavity. Thus, the cell migration from the primary tumor into the abdominal organs and the lungs followed different paths as compared with the second series. The fourth series provided data obtained with non-colloidal but particulate radioisotope-chromic phosphate.

The records of Table I show that in several groups of each series the incidence of growth from inoculated organs was invariably lower for treated animals than for controls. In experiments with colloidal radiogold which is known to be stored mainly in the liver(7), clear cut results were observed in animals inoculated either into the peritoneal or the pleural cavity and treated intravenously. Similarly in experiments with intravenously injected suspensions of chromic phosphate, lower frequency of tumor growth was recorded mainly in animals injected with lungs which previously were found to intercept large particles of $CrPO_4$(8). Local treatment of primary tumor growth with colloidal radiogold did not reduce significantly the frequency of tumor growth induced by the liver, (first series). All these data indicate that the storage of a radioisotope in the organ of a tumor bearing mouse was a prerequisite for the several instances of failure of growth from this organ after intravenous treatment. The most marked results were obtained by giving the treatment on the day of primary inoculation or shortly (1 or 2 days) before or after. The independence of this effect from the route of tumor cells spread into organs is illustrated by similarity of results in the second and the third series.

The 10-days period of observation was accepted because previous work(1) has shown that this interval provided most consistent results. However some of mice negative on examination after 10 days developed peritoneal growth 20 to 40 days later. This was attributed to the presence in injected material of tumor cells in very small numbers of very low viability, etc.

Discussion. Since only traces of radioactive material (considering its dilution in the body and its decay after 3- to 5-day interval between inoculation and autopsy) were introduced in the course of assay implantation with the organ brei of treated mice into mice recipients, the delay in tumor growth can be attributed to damage by intravenously injected radioisotopes to metastatic tumor cells in these organs. Whether this damage consisted in inhibition of mitoses or advanced disintegration of the cell structure, it implied, according to our results, loss or attenuation of viability of a certain number of tumor cells.

Previous work has shown(3,4,9,10) that free tumor cells are more vulnerable to radiation and to some physical and chemical agents than organized tumor structure. This may account for the deep damage inflicted in our experiments to free metastatic cells by radioisotopes in doses well tolerated by animals. It may also explain the importance of early treatment (not more than 2 or 3 days before or after inoculation) *i.e.* before metastatic cells intercepted by organs had sufficient time to elicit vascularization and organized growth. This interpretation may suggest the use of intravenous injections of suitable radioisotopes immediately before or after surgical intervention on certain tumors in order to induce at once damage to metastatic cells spread into the liver or the lungs from manipulation during operation.

Summary and conclusion. 1. Intraperitoneal inoculation of liver brei from sarcoma-37 (i.p. growth, 5-day-old) induced tumor growth in nearly all of mice. This was attributed to the presence in the liver of metastatic cells spread from the primary tumor. Similar results were obtained only in rare instances with liver brei from the mice of the same series injected intravenously with colloidal Au^{198} on the date of primary tumor inoculation (graft) or 2 days earlier or later. Analogous difference in results was recorded in experiments with i.p. inoculation of lung brei from tumor-bearing mice untreated and treated intravenously with radioactive chromic phosphate. It was concluded that injected radioisotopes reduced the number and the viability of metastatic tumor cells in the liver or in the lung. 2. Tumor growth from the organs which retain only small amounts of particulate isotopes (spleen) or none was but slightly inhibited or not at all. It was concluded that retention of radioisotopes by the organ was a prerequisite for their effect on metastatic tumor cells in this organ. 3. The possibility to induce damage to metastatic tumor cells without inflicting similar damage to normal tissues of the host was attributed to high vulnerability of metastatic cells as free cells (*i.e.* before their organized growth). 4. It is suggested that the method reported above may be useful for screening the activity of radioactive and chemical agents against metastatic tumor cells in organs of the mouse, and it is presumed that in certain clinical conditions it may be attempted to attack selectively metastatic tumor cells by suitable radioisotopes.

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Complement Fixation with Leptospiral Antigens. (20708)

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(Introduced by S. Edward Sulkin.)

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Although there have been indications for many years that a complement fixation test may be a practical procedure in the diagnosis of leptospirosis*(1-5), it has not received general acceptance. Current interest in complement fixation in this country was first stimulated by the report of Randall *et al.*(6) which described the preparation of an antigen by means of sonic disintegration of washed leptospirae. Such antigens have been shown to have diagnostic value but tend to cross react with sera for heterologous types(7,8). The supernatant fluid of leptospiral cultures was later shown to contain a relatively type-specific soluble antigen that fixed complement in the presence of hyperimmune rabbit sera(9). More recently Schneider(10,11) has obtained by chemical fractionation of washed leptospirae a complement fixing antigen which shows a wide range of cross reaction with heterologous leptospiral antisera. In a complement fixation test for leptospirosis in cattle York(12) has used an antigen consisting of a heavy suspension of *Leptospira pomona* cultivated in chick embryos.

In the present investigation we have attempted to compare the antigenicity of several leptospiral preparations as determined by complement fixation tests with sera of immunized rabbits.

Materials and methods. Stock cultures of leptospirae† were maintained in Korthof's medium(13) at room temperature with transfers every 2 to 3 weeks. In some instances the leptospiral concentration in cultures and allantoic fluid was determined by direct microscopic count using a Petroff-Hauser counting chamber. Rabbits were immunized with live cultures giving intravenous injections at 5-day intervals starting with a dose of 1 ml and increasing to 5 ml for the last injection. *The agglutination-lysis test* was performed by adding 0.1 ml of culture to 0.1 ml of each serum dilution. Serial doubled dilutions of serum were made in saline. If necessary, cultures were diluted with Korthof's medium to contain about 50 organisms per high dry darkfield. After incubation of the mixtures for 2 hours at 37°C they were examined under the darkfield microscope. The highest dilution of serum showing definite clumping was recorded as the titer. *Complement fixing* activity of antigens was determined by combining 0.2 ml of antigen dilution, 0.2 ml of a 1:20 dilution of immune serum inactivated at 62°C for 40 minutes and 0.2 ml of fresh guinea pig serum dilution containing 2 units of complement. After fixation overnight at 6-8°C, 0.4 ml of hemolytic system containing 2 units of hemolysin was added and the tests were read after 30 minutes incubation at 37°C. The reciprocal of the highest dilution of antigen showing complete fixation was recorded as the titer.

Results. Culture fluid antigens. Numerous 14- and 28-day cultures of 8 types of leptospirae in Korthof's medium were killed by the addition of merthiolate, 1:10000, and were centrifuged at 15000 r.p.m. (Spinco) for 30 minutes. The majority of these undiluted supernatant fluids completely fixed complement in the presence of homologous immune serum; a few gave titers of 2 but none showed a titer greater than 4. These fluids were frequently anticomplementary in a dose of 2 antigenic units even after heating at 62°C for 40 minutes. The supernates of cultures grown for 28 days tended to be more antigenic than those from 14-day cultures suggesting that the autolysis which occurs as cultures age

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* The early literature is reviewed in the monograph by Borgen(1).

† Strains of *L. icterohemorrhagiae*, *L. canicola*, *L. pomona*, and *L. grippotyphosa* were kindly supplied by Dr. P. B. Beeson, Emory University Medical School, Atlanta, Ga. *L. autumnalis*, type AB (*L. akiyama A*) and *L. bataviae* (Swart v. Tienen) were obtained from Dr. E. H. Thomas, National Microbiological Institute, Bethesda, Md.

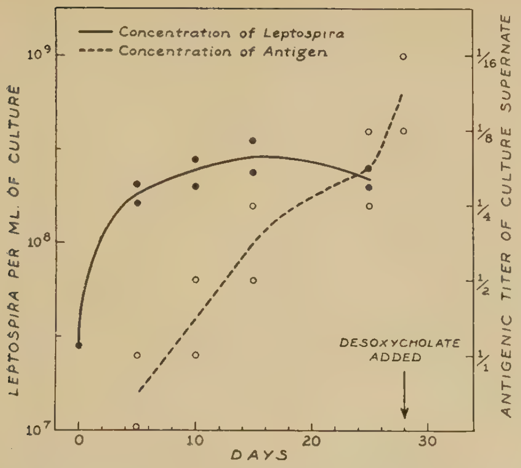

FIG. 1. Antigen concentration in culture supernate in relation to growth of *Leptospira pomona*.

may contribute to the antigenicity. In 3 experiments in which duplicate cultures of *L. icterohemorrhagiae* and *L. pomona* were grown at 28° and 37°C for 14 days the antigenic titer of the supernatant fluid of the 37°C culture was twice that of the 28°C culture in spite of the higher concentration of leptospirae at the lower temperature as demonstrated by actual count. This difference was presumably due to the increased autolysis at 37°C(14). We were not able, however, consistently to prepare sufficiently antigenic supernates for practical use even when a long period of incubation at 37°C was employed. To explore further the relation of soluble antigen concentration to the concentration of organisms leptospiral counts and soluble antigen determinations were made on samples removed at intervals from duplicate cultures of *L. pomona* grown at 28°C (Fig. 1). The number of leptospirae per ml of culture reached a maximum in about 15 days and then declined. The antigenic titer of this supernate continued to increase while the concentration of organisms was decreasing, again suggesting the liberation of antigens as a result of autolysis. In order to bring about further lysis sodium desoxycholate(10) to give a concentration of 0.2% was added to the culture after 28 days of incubation. After 1 hour at 37°C and 18 hours in the refrigerator the cultures were centrifuged and the supernatant fluid dialysed against isotonic phosphate saline solution(15). This treatment resulted in a still further increase in antigenic titer of the culture fluid.

Antigens from chick embryos. Six types of leptospirae were passed from 11 to 16 times through 8-day-old embryonated eggs. Allantoic fluid was harvested 5 days after inoculation and 0.5 ml transferred to the allantoic cavity of 8-day-old embryos. The concentration of organisms in the allantoic fluid reached a level comparable to that obtained in cultures, 100000000 to 200000000 per ml in most eggs. Since leptospirae failed to multiply in some eggs, however, it was necessary to check the allantoic fluid from each egg by darkfield. After high speed centrifugation, infected allantoic fluid fixed complement weakly if at all. This low concentration of soluble antigen was probably due to the fact that little autolysis took place in the relatively brief period of incubation. The isolation of a serologically active substance from typhus rickettsiae by boiling infected yolk-sac material in dilute alkali(15) suggested applying this procedure to leptospira-infected allantoic fluid. The pooled fluid from several eggs was centrifuged at low speed to sediment erythrocytes and debris. The supernatant fluid was made 5th normal with sodium hydroxide, placed in boiling water for 30 minutes, and neutralized by dialysis against buffered saline(15). The complement fixing titer of this dialysed material was determined in the presence of homologous immune rabbit serum. Of 13 preparations from 6 types of leptospirae, 6 had antigenic titers of 16, 1 had a titer of 8, 4 had titers of 4 and 2 had titers of 2. Only one of the preparations was anticomplementary in a dose of 2 units. There was a rough correlation between concentration of leptospirae in the fluid and the antigenic titer after treatment with alkali. Attempts to obtain agglutination of erythrocytes treated with these preparations have been completely unsuccessful.

Specificity of antigens. Table I shows the complement fixing titers of homologous and heterologous rabbit immune sera for 4 antigenic preparations from *L. pomona* as compared to the agglutinin titers of these sera. Each serum agglutinated its homologous type to a titer of at least 20480. The complement fixing antigens were used in a dose of 2 units provided by the dilutions indicated. The

TABLE I.
Specificity of *L. pomona* Complement Fixing Antigens as Compared with Agglutination.

Antigen	Titer of rabbit immune serum* 1	2	3	4	5	6	7	8
Whole culture (agglutination)	0†	0	81920	0	0	0	0	0
Culture supernatant fluid, undiluted	0‡	0	640	0	0	0	0	0
Supernatant fluid of culture treated with desoxycholate, 1:8	0‡	0	320	0	0	0	0	0
Allantoic fluid treated with NaOH, 1:8	0‡	0	160	0	0	0	0	0
Washed suspension of leptospirae from allantoic fluid, 1:16	0‡	0	640	0	0	0	0	0

* 1. *L. icterohemorrhagiae*, 2. *L. canicola*, 3. *L. pomona*, 4. *L. hebdomadis*, 5. *L. grippotyphosa*, 6. *L. mitis*, 7. *L. bataviae*, 8. *L. autumnalis*.

† No agglutination in a 1:80 dilution of serum.

‡ Incomplete complement fixation in a 1:20 dilution of serum.

washed suspension from allantoic fluid was prepared according to the method of York (12). Under these conditions none of the antigens showed complete fixation with any of the heterologous sera tested.

Discussion. Soluble antigens that fix complement have been prepared from leptospirae by several methods. Although there is as yet no proof of their identity, it is possible that the antigen present in untreated culture supernate, the antigen extracted from the organisms by treatment with desoxycholate, Fraction 1 of Schneider(10), and the antigen present in sodium hydroxide treated allantoic fluid are the same. The antigenic preparations from *L. pomona* studied here exhibited a high degree of type specificity. Antigens prepared by sonic disintegration(6,8) and by chemical fractionation(11), however, are reported to have a broad range of reactivity among the various types of leptospirae. In an extensive experience with leptospiral suspensions as complement fixing antigens Gaehtgens(4) observed cross reactions but he found the homologous titer always to be higher. Further work may reveal to what extent these apparent differences in type specificity may be due to quantitative differences in the reactivity of preparations or to variations in the cross reactivity of different strains.

Summary. The complement fixing activity of preparations from leptospiral cultures and from leptospira infected chick embryos has been studied in the presence of immune rabbit serum. The antigenicity of culture supernatant fluids was low but tended to increase as the temperature of incubation and the age of the cultures increased. The addition of sodium desoxycholate to cultures increased still further the ability of culture supernates to fix complement. Boiling infected allantoic fluids with N/5 NaOH yielded a soluble, non-dialyzable antigen. *L. pomona* culture supernates, alkali treated allantoic fluids and washed suspensions of leptospirae from allantoic fluids reacted specifically with *L. pomona* immune rabbit serum in the dilutions employed.

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Antagonist Studies: Comparison of Desoxypyridoxine and Pyridoxine-Deficiency Therapy on Various Vitamin B_6 Enzymes.* (20709)

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The use of antagonist therapy to duplicate or accentuate a vitamin deficiency is a common practice. Many anti-metabolites produce gross and histological changes similar to, if not identical with, those observed on a deficient regimen. Although many investigators have studied the effect of vitamin deficiencies on enzymatic systems normally containing the lacking metabolite, little work has been done pertaining to the action of vitamin antagonists on these same systems. The stimulus for this study arose from the observation that differential toxic effects on cancer and host tissue can be best obtained if tumor-bearing animals are permitted *ad libitum* access to a complete diet while on antagonist therapy. Although it is appreciated that quantitative biochemical differences between normal and cancer cells may for the most part explain this phenomenon, the successful carcinostatic results reported with pyridoxine and riboflavin antagonists(1-4), without recourse to metabolite-deficient diets, raise further question to the belief that identical biochemical lesions are produced by antagonist therapy and metabolite-deficiency therapy.

The present communication reports a comparison of the effect of vit. B_6 antagonist, desoxypyridoxine, and a vit. B_6 deficiency on 3 vit. B_6-containing enzymes in the mouse.

Methods: C57 male black mice from 6-8 weeks of age were employed throughout. A total of 80 mice were used in these studies. Twenty mice were treated with the vitamin antagonist and 20 were held as controls. Twenty mice were placed on the vit. B_6-deficient diet and the remaining 20 received the identical diet plus the vitamin. The desoxypyridoxine-treated mice received an *ad libitum* diet of Rockland chow and water as did their saline controls. The vit. B_6 antagonist was administered intraperitoneally at a level of 150 mg/kg body weight per day for a 14-day period. Under these conditions, no loss of weight or other outward signs of a vit. B_6 deficiency were observed. Each control animal received 0.25 ml of saline daily. All animals were sacrificed one hour after the final injection. The mice used in the deficiency studies were housed in plastic cages with raised screen floors and received a high protein diet consisting of: vitamin-free casein 50, salt mixture 4(5), Cerelose 40, corn oil 5 g; cod liver oil (4500 U.S.P. units of vit. A and 450 U.S.P. units of vit. D per g) 250, choline chloride 150, calcium pantothenate 4.0, niacin 4.0, riboflavin 0.6, thiamine hydrochloride 0.6, inositol 20.0, biotin 0.03, folic acid 0.02, and 2-methyl-1,4-napthoquinone 0.04 mg. Control animals received an identical ration except that 0.6 mg of pyridoxine hydrochloride was included. All animals on the deficient regimen lost weight, an average decrease of 40% of the initial weight being observed for the 4-week experimental period. No significant enzymatic differences were noted between control animals receiving the pyridoxine-supplemented diet and mice fed a stock chow diet. Throughout the experimental period the food consumption of the control animals in the vit. B_6 deficiency studies was restricted to a level comparable to that ingested by the deficient animals. Water was supplied *ad libitum*.

All animals were killed by cervical rupture, the livers being immediately removed and chilled in ice cold saline. The tissues were then blotted dry and weighed. Portions of each liver were taken and analyzed for glu-

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† Fellow of the Dazian Foundation for Medical Research.

TABLE I. Effect of High Protein-Induced Vit. B_6 Deficiency on Liver Enzymatic Activities.*

Systems	Control	Deficient
Transaminase†	229 ±13	143 ±10
Dopa decarboxylase‡	1.37± .07	1.16± .03
Cysteine desulfhydrase§	8.2 ± .7	4.2 ± .5

* Values are the avg of 8 individual observations, run in duplicate and include the stand. error.

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

† Reaction mixture consisted of 0.1 ml of a 0.5% homogenate, 0.5 ml 0.67 M L-aspartic acid, 0.1 ml 0.1 M alpha-keto-glutaric acid and 0.1 M phosphate buffer (pH 7.4) to 2.0 ml final vol; incubated 10 min. at 37°C. See text for the definition of units.

‡ Reaction mixture consisted of 0.5 ml of a 10% homogenate, 0.3 ml 0.1 M DL-dopa, 0.05 ml octyl alcohol and octyl alcohol-saturated phosphate buffer (0.02 M, pH 6.9) to give a final vol of 2 ml; incubated 15 min. at 37°C. See text for definition of units.

§ Reaction mixture consisted of 2 ml of super natant fluid equivalent to 20% homogenate in 0.1 M phosphate buffer (pH 7.4) and 1 ml of 0.03 M L-cystine; incubated for 2 hr at 37°C. Values are expressed as micromoles of ammonia nitrogen liberated per 2 hr per g tissue wet wt.

tamic-aspartic transaminase, dihydroxyphenylalanine (dopa) decarboxylase and cysteine desulfhydrase using the methods of Tonhazy *et al.*(6), Dietrich(7), and Greenstein *et al.* (8), respectively. L-cystine was used as the substrate in the cysteine desulfhydrase studies. Transaminase and dopa decarboxylase activities are reported as microliters of CO_2 liberated per mg tissue dry weight per 10 minutes (Q_T^{10}) and 15 minutes (Q_T^{15}) incubation period, respectively. Cysteine desulfhydrase activity is reported as micromoles of ammonia liberated per 2 hours per g tissue wet weight. All determinations were conducted at 37°C and at least 8 individual determinations were carried out per group.

Results. It has been demonstrated by other investigators, employing growth(9) and tissue analyses(10) as criteria, that a high protein diet accelerates the depletion of the vit. B_6 reserves in animals receiving a vit. B_6-deficient diet. The effect of such a regimen on the activity of 3 vit. B_6-containing enzyme systems (transaminase, dopa decarboxylase and cysteine desulfhydrase) is summarized in Table I. The activity of all 3 systems is significantly lowered, cysteine desulfhydrase being the most sensitive to vit. B_6 deprivation, followed by transaminase and dopa decarboxylase. These results are similar to those obtained by other investigators in other species(11-14).

The effect of desoxypyridoxine on transaminase, dopa decarboxylase and cysteine desulfhydrase activity is presented in Table II. Desoxypyridoxine administered at a level of 150 mg/kg body weight to mice receiving an *ad libitum* diet containing ample vit. B_6 was observed to severely antagonize transaminase activity. Dopa decarboxylase was antagonized to a lesser but still significant extent. Cysteine desulfhydrase activity was, however, uninhibited.

The participation of pyridoxal phosphate as a prosthetic group in the 3 enzymatic systems studied has been demonstrated beyond reasonable doubt. The metabolically active form of desoxypyridoxine, desoxypyridoxine-5-phosphate, has been shown by Umbreit and

TABLE II. Effect of Desoxypyridoxine Therapy on Liver Enzymatic Activities.*

System	Saline control	Desoxypyridoxine-treated
Transaminase†	210 ±27 (8)	108 ±45 (8)
Dopa decarboxylase‡	1.12± .07 (14)	.92± .03 (14)
Cysteine desulfhydrase§	7.3 ± .7 (15)	9.5 ± .8 (15)

* Figures in parentheses represent number of individual observations. Values are avg of duplicate determinations and include stand. error.

$$\sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

† Reaction mixture consisted of 0.1 ml of a 0.5% homogenate, 0.5 ml 0.67 M L-aspartic acid, 0.1 ml 0.1 M alpha-keto-glutaric acid and 0.1 M phosphate buffer (pH 7.4) to 2.0 ml final vol; incubated 10 min. at 37°C. See text for the definition of units.

‡ Reaction mixture consisted of 0.5 ml of a 10% homogenate, 0.3 ml 0.1 M DL-dopa, 0.05 ml octyl alcohol and octyl alcohol-saturated phosphate buffer (0.02 M, pH 6.9) to give a final vol of 2 ml; incubated 15 min. at 37°C. See text for definition of units.

§ Reaction mixture consisted of 2 ml of supernatant fluid equivalent to 20% homogenate in 0.1 M phosphate buffer (pH 7.4) and 1 ml of 0.3 M L-cysteine; incubated for 2 hr at 37°C. Values are expressed as micromoles of ammonia nitrogen liberated per 2 hr per g tissue wet wt.

Waddell(15) to compete with pyridoxal phosphate for the apoenzyme. The degree to which desoxypyridoxine will antagonize a pyridoxal phosphate-containing system is for the most part affected by the amount of pyridoxal phosphate available and the dissociation constant of the coenzyme-apoenzyme complex. Under the conditions of the experiment the amount of pyridoxal phosphate present can be assumed to have remained relatively constant. Thus, the only remaining variable of consequence is the dissociation constant of the various vit. B_6-containing systems. Since this variable is also the main factor affecting the rate at which enzymatic activity is depleted through vitamin deprivation, one might expect similar effects on enzymatic systems containing pyridoxine, regardless of whether these systems are antagonized by desoxypyridoxine or a pyridoxine deficiency.

Experimentally, however, this is not the case. Transaminase and dopa decarboxylase behave according to expectation. Cysteine desulfhydrase, although very sensitive to vit. B_6 deprivation, is not antagonized by desoxypyridoxine therapy.

This latter phenomenon is not understood. The direct participation of desoxypyridoxal phosphate as an activator in the cysteine desulfhydrase system is doubtful in view of the work of Meister *et al.*(14), since these investigators observed a marked depression of cysteine desulfhydrase activity in vit. B_6-deficient rats receiving desoxypyridoxine. It is possible that pyridoxal phosphate combines with the protein moiety of cysteine desulfhydrase in such a manner as to make the configuration at the 4 position of the pyridine nucleus critical in regard to apoenzyme-prosthetic group affinity. If this were the case, replacement of the aldehyde by a methyl group at this position could produce a compound totally ineffective in displacing pyridoxal phosphate in the cysteine desulfhydrase system. Desoxypyridoxine phosphate, on the other hand, can displace pyridoxal phosphate bound to the apoenzyme of either transaminase or dopa decarboxylase; apparently, in these cases the aldehyde grouping at position 4 is essential for enzymatic action. This is in keeping with the postulation that the first step in transamination and decarboxylation reactions is a Schiff base formation involving the pyridoxal moiety of the enzyme and the amino acid concerned(16,17). The observation of Heyl *et al.*(18) that L-cysteine reacts with a pyridoxal to form a thiazolidine compound, rather than the Schiff bases produced by most other amino acids, would tend to exclude the involvement of a Schiff base reaction in the enzymatic degradation of cysteine. The stability of cysteine desulfhydrase preparations in the presence of substrate, together with the low degree of dissociation of the thiazolidine compound formed by the reaction of pyridoxal with L-cysteine(18), permits the surmise that very little of this material is formed in the presence of cysteine desulfhydrase, and may indicate that the pyridoxal phosphate is bound to the apoenzyme of cysteine desulfhydrase in such a manner as to make the aldehyde group unavailable.

These results present the possibility that vitamin antagonists may be synthesized to function solely as specific anti-enzymes, and not as antagonists for all the biochemical functions of the vitamin. This study accentuates the well-recognized potentialities of anti-metabolites as biochemical tools capable of more clearly defining pathways of intermediary metabolism. More important, perhaps, the data present the possibility that vitamin antagonists may be useful as highly precise chemotherapeutic agents, particularly in the presence of a normal dietary intake.

Summary. 1. Mice receiving a vit. B_6 deficient-high protein diet were observed to have significantly lower transaminase, dopa decarboxylase and cysteine desulfhydrase activities as compared to similar animals receiving an identical diet supplemented with pyridoxine. Cysteine desulfhydrase was the most sensitive to vit. B_6 deprivation, followed by transaminase and dopa decarboxylase. 2. The administration of desoxypyridoxine to mice receiving an *ad libitum* stock diet containing adequate vit. B_6 significantly decreased transaminase and dopa decarboxylase activity. However, cysteine desulfhydrase activity was uninhibited. 3. A possible explanation of the observed results is discussed,

the potentialities of vitamin antagonists as precise chemotherapeutic agents being indicated.

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Poliomyelitis Virus in Tissue Culture VI. Use of Kidney Epithelium Grown on Glass.* (20710)

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Tissue culture methods have become essential to the conduct of research with poliomyelitis virus and indeed with certain other viruses as well(1). For this purpose rolled or stationary test-tube cultures of tissue have been chiefly used in which the tissue fragment is imbedded in a plasma clot. This paper reports a simplification of the technic for preparing cultures, in that the tissue fragment can be made to stick on glass in the absence of plasma and clotting solution (chick embryo extract or thrombin). The new cells then grow out directly on the glass surface. Before the culture is used, the tissue fragment, easily removed to leave behind a sheet of newly grown cells, can be transferred to seed another culture tube. A variety of tissues from man, monkeys, and mouse have been cultured on glass without plasma. However, this paper will deal exclusively with the culturing and use of monkey kidney tissue.†

Materials and methods. The technic of preparing test tube cultures of monkey tissue in plasma as used in this laboratory has been described in detail(4,5). Similar conditions have been followed in the present experiments in preparing the plasma-clot test-tube cultures, (hereafter called "plasma tubes"). Test tube cultures prepared in the absence of plasma are called "dry tubes", and their preparation is given in detail below.

Complete medium for all cultures, in the presence or absence of plasma clot, consisted of 9 parts of mixture No. 199(6) and 1 part bovine serum.‡ The No. 199 was made up in

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* Aided by a grant from the National Foundation for Infantile Paralysis.

† Epithelial and fibroblastic cells derived from human cancer and capable of growth on glass are also sensitive to poliomyelitis virus(2,3). For certain purposes where one does not want a cell line of cancerous origin, or a cell which requires human serum for its growth, or a cell line which may suddenly be lost because of mold or other contamination, the use of primary explants from readily available normal tissue offers advantages.

Earle's salt solution containing 2.20 g of $NaHCO_3$ per liter. Penicillin and streptomycin were added so that the final concentration in the complete medium was 100 units and 100 μg per ml respectively. All tubes contained 1 ml of complete medium, and were incubated at 36°C in a stationary position(5). When in 10-14 days an epithelial sheet of cells had grown out, the complete medium was removed, the tubes were washed with 1 ml of Earle's salt solution (to remove poliomyelitis virus inhibitory substances present in bovine serum), and to each tube there was added 0.09 ml of maintenance medium consisting of 1 part of No. 199 and 1 part of Earle's salt solution. The cultures were then ready for use with virus. In many experiments, the original tissue fragments were shaken loose after the outgrowth was sufficiently luxurious, and they were transferred together with the original complete medium to a new test tube. During the manipulation, the outgrowth from the tissue remained attached to the wall of the tube. The original tube was now washed and maintenance medium added as indicated above. The subculture containing the original fragments and fluid was fortified with 0.25 to 0.5 ml of fresh complete medium, and incubated also at 36°C in a stationary position. The Brunhilde (Type 1) and Y-SK (Type 2) poliomyelitis viruses in their 11th and 21st tissue culture passages were employed. Tubes were read every 24 or 48 hours for 6 days for their reaction to virus (cytopathogenic effect). *Test tubes* (16 x 150 mm) are immersed overnight in "7 x" detergent solution[§] diluted 1 to 10 with tap water. The following morning the tubes are taken from the detergent solution and placed in groups of 170 in stainless steel boxes (256 mm x 156 mm x 156 mm deep) with the open ends at the top. The tubes are scrubbed with a test tube brush while they are in the box. As an aid in rinsing the tubes, a wire grid cover is employed. With the cover in place the box and tubes are flooded with tap water. The tubes are rinsed 10 times in tap water and 3 times in distilled water. A paper liner is fitted into the cover and the box and tubes are autoclaved at 15 lb for 30 minutes, and then dried at 105°C for 3 hours. In the afternoon these tubes are placed in a 45°C incubator for the following day's production of cultures. *Cultures in "dry" tubes.* Soon after its removal from a cynomolgus or rhesus monkey, the kidney is minced in a widemouth tube with 2 volumes of Earle's solution, using conventional sharp scissors, or Bard-Parker replaceable-blade scissors. The kidney is reduced to fragments of about 1 mm, which are transferred to a flask and washed by repeated addition of salt solution and decantation of the turbid supernate, using about 300 ml of salt solution per kidney. The washed fragments are kept under 50 ml of salt solution at 4°C until needed, but for not longer than 3 days. One pair of kidneys from a young monkey (5 to 7 lb) will provide tissue for 2000 primary culture tubes. Warm tubes from the box are placed on a rack in groups of 24. A narrow line of kidney fragments is streaked into the lower third of the tubes, with the minimum amount of fluid necessary to make the streaking possible. An uncalibrated pipette with a small rubber bulb is used to take up enough tissue from the flask at one time to prepare a full rack of 24 tubes. The tissue-containing tubes are then transferred into another box. With 2 persons working together the preparation of 170 tubes in this manner takes about 5 minutes. During this time there is a drop in temperature of the tubes from the original 45°C. Whatever this may amount to, the technic is not so sensitive that the adhesion to the tubes or growth potential of the tissue varies discernibly in the first or last tubes made in each box. When the 170 tubes have all been placed in the second (transfer) box, the sterile top from the first box is also transferred to it. These tubes remain at room temperature for about 5 minutes while the next box is being prepared. After this time, they are transferred to a refrigerator (4°C) where they remain for 30 minutes. 1 ml of complete medium is then added by an automatic hand pipette, care being exercised not to direct the fluid on the tissue fragments. The tubes are

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[‡] Bovine serum was obtained from Microbiological Associates, Bethesda, Md. It was passed through a bacteriological filter and then stored and shipped in the frozen state.

[§] Obtained from Linbro Chemical Co., New Haven, Conn.

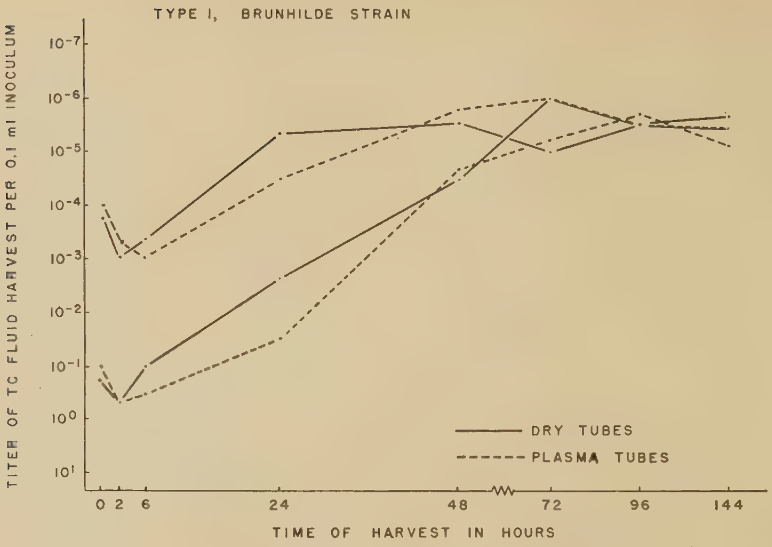

FIG. 1. Growth curves of the Brunhilde Strain (Type 1) in kidney epithelial cells grown in presence and in absence of a plasma clot.

quickly stoppered after addition of medium, and transferred to a new box similar in size to those described above. The storage box has a strip of board taped across the front lower edge to provide the desired tilt to the tubes (about 5°). A row of small nails with 4 mm heads driven into the back of the box 16 mm apart and 12 mm from the bottom of the box keeps the first row of tubes in position and facilitates the storage of the upper rows. Each box firmly holds 145 tubes. Each alternate tube is pulled out about ½ inch to reduce excessive tilting of the upper rows (which would otherwise result from the diameter of the stopper being larger than that of the tube). The tubes are left *undisturbed* at 36°C for 10 to 14 days, at which time clear luxuriant sheets of epithelial cells averaging 10 mm in mean diameter appear. Not every tissue fragment gives rise to a sheet of cells, for usually 2 to 4 cellular sheets are formed in each tube. The percentage of usable tubes (having cellular sheets of 8 mm or greater) varied from 70 to 100%.

Virus development in plasma and dry tubes. A comparison of the capacity of the two types of culture tubes to support virus growth was made by inoculating groups of each with high and low dilutions of Types 1 and 2 poliomyelitis virus and harvesting the tissue-culture fluids at intervals over a period of 6 days. The harvested fluids were frozen at –20°C. When all of the fluids had been collected they were titrated simultaneously, using serial tenfold dilutions. The Brunhilde strain of Type 1 was titrated at the time of inoculation and yielded a TC_{50} of 10^{-6} per 0.1 ml of inoculum in plasma tubes and $10^{-5.8}$ in dry tubes. The Y-SK strain of Type 2 was similarly titrated at this time and had a titer of 10^{-5} in plasma tubes and $10^{-5.3}$ in dry tubes. The stock viruses were arbitrarily diluted to give about 100,000 and 100 TC_{50} doses for Brunhilde and 10,000 and 10 TC_{50} doses for Y-SK virus. Intervals of 2, 6, 24, 48, 72, 96, and 144 hours were chosen for harvesting. Eight tubes of each type, plasma and dry, were taken at each collection period for each of the inoculated series, making a total of 64 tubes for each harvest. The tissue-culture fluids from the 8 tubes of each set were pooled into 2 groups of 4 tubes each. Each dilution in the titration series was inoculated into 4 culture tubes which were then followed for 6 days. The cytopathic changes usually became apparent in 2 to 3 days with large doses of virus and in 4 to 5 days with low doses. As shown in Fig. 1 and 2, there appeared to be no significant difference between the curves obtained with dry and plasma-clot tubes nor between those for the

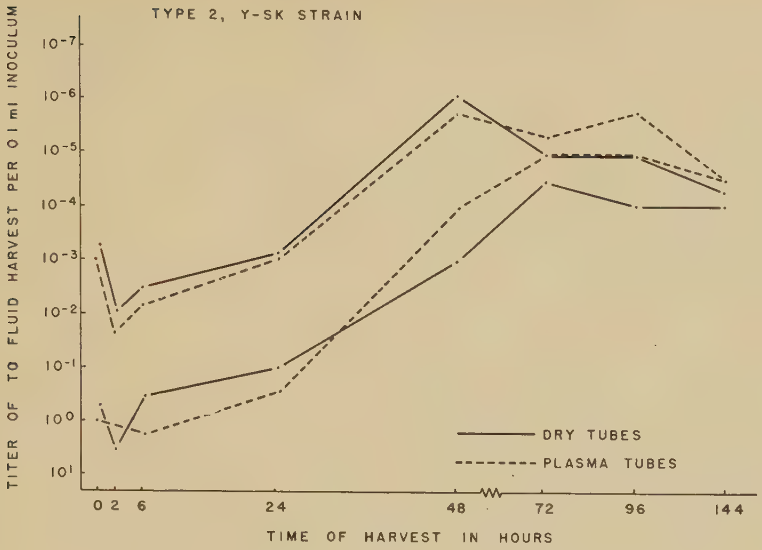

FIG. 2. Growth curves of Y-SK Strain (Type 2).

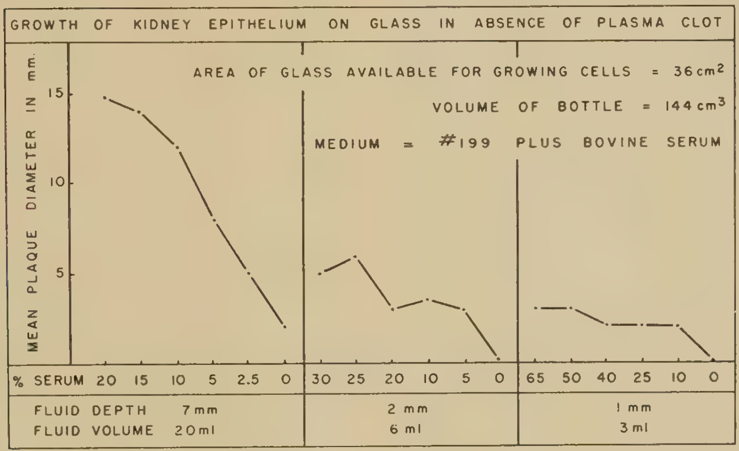

FIG. 3. Effect of serum concentration and fluid volume (or depth) on growth of kidney epithelial cells on glass.

2 strains of virus employed. The initial drop in titer was approximately 90%. Peak titers were reached in 24 to 48 hours in tubes receiving the high virus dose, whereas 72 hours were required for the more dilute viruses to attain corresponding titers. Cytopathic changes occurred at about the time the peak titers were observed. The highest titer observed was in the Y-SK series where the 48 hours sample rose to concentrations of 10^{-6} per 0.1 ml, in both dry and plasma clot tubes. When low concentrations of virus were used as inocula, virus could not always be detected in the standard 0.1 ml inoculum, even when undiluted fluids were tested. Therefore, in order to test for virus in such fluids, the sample being tested was used to replace the complete medium in the culture tubes of the test. In this way 1 ml of inoculum was introduced into the culture. If 2 of 4 tubes thus inoculated became positive, the titer was expressed as 10^1.

Dry technic in bottle cultures. The same technic employed in making dry tubes can be used in growing plasma-free cultures in bottles.

TABLE I. Growth of Tissue Following Transplantation from 12- to 14-Day-Old Cultures Prepared in "Dry" Tubes.

Group	Medium in transplanted tubes	Total No. of tubes with primary outgrowths	Secondary outgrowth of epithelial cells							Day of reading secondary culture
			Good growth		Min growth		% with growth	No growth		
			No.	%	No.	%		No.	%	
A 1	Transfer from original tube	82	18	22	23	28	50	41	50	4
2	Original + 0.25 ml fresh medium	46	36	78	2	5	83	8	17	4
3	Original + 0.5 ml fresh medium	92	74	80	6	7	87	12	13	4
B 1	Transfer from original tube	120	86	72	6	5	77	28	23	7
2	Original + 0.3 ml fresh medium	110	90	82	0	0	82	20	18	7

The amount of tissue needed varies with the size of the bottle. For example with a bottle having a flat side of 36 sq. cm., 4 or 5 drops of minced kidney suspension are placed on the inside wall and spread about with a pipette. The bottle is stoppered and turned to allow excess fluid to drain from the tissue. After 5 minutes at room temperature and ½ hour at 4°C, the complete medium is added to cover the tissue with about 7 mm of fluid. The growth rate of monkey kidney tissue in bottles parallels that of test tube cultures. Fig. 3 illustrates a typical experiment. Bovine serum was an important constituent of the medium; concentrations of 10%, when used with No. 199, were satisfactory for good outgrowth. Of the three depths of fluid studied in the experiment, that of 7 mm was found to give the best growth. Subsequent experiments have shown that with deeper layers of fluid (up to 20 mm) growth may be enhanced even further. Prior to inoculation of bottle cultures, tissue fragments are manually shaken loose from the wall of the bottles. The fragments and the media in which they are suspended are transferred to another sterile bottle and incubated further at 36°C. The original bottle is rinsed thoroughly with salt solution to wash out the bovine serum, and maintenance medium (equal parts of No. 199 and Earle's salt solution) is added to cover the tissue with about 7 mm of fluid. Bottles with confluent growth inoculated with 10,000 to 100,000 TC_{50} doses of virus yielded harvests 24 to 48 hours later with titers of $10^{-5.5}$ to $10^{-6.5}$, and at times even 10^{-7} per 0.1 ml. This has occurred with strains of all three types: Brunhilde and W-S (Type 1), Y-SK (Type 2) and Leon (Type 3). The fragments which have been transferred to the second bottle react differently from fresh fragments. They settle spontaneously in the presence of the complete medium, attach, and, apparently being predisposed to grow, reach satisfactory proportions in 6 to 8 days. The fragments may then be used for a second transfer, but not beyond this point because of their greatly reduced size. The original medium is transferred along with the fragments. However, it has been advisable on the second transfer to recharge it with ¼ to ½ volume of fresh medium. Additional experiments on the re-use of kidney fragments in the production of test tube cultures is given below.

Cultures made from transplanted fragments.[‖] Several thousand secondary cultures, and even tertiary cultures, have been prepared by the technic outlined under *Methods.* The addition of ¼ to ½ volume of fresh medium usually increases the number of useful tubes obtained (as in Groups A1, A2, and A3 of Table I) but this is not always true (as in Groups B1 and B2). The yield of tubes in the secondary cultures is of the same order of magnitude as in the primary cultures, about 75 to 90%. Simultaneous titrations of a number of virus samples of all 3 types were made in primary and secondary cultures, and there was no significant difference in the observed results (Table II).

Discussion. Culturing fragments of kidney tissue on glass without a plasma clot has many advantages over the technic with plasma. Cultures can be prepared more easily and more rapidly. The clotting system of plasma and chick embryo extract is no longer needed, nor

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[‖] Dr. Emanuel Eylan, Fellow of the World Health Organization, on leave from the Ministry of Health, Jerusalem, Israel, actively participated in these experiments during a brief visit to New Haven.

TABLE II. Replicate Titrations of Virus in Primary, Secondary, and Tertiary Epithelial Outgrowths.

Virus	Titer of virus (negative logarithm) in indicated culture		
	Primary	Secondary	Tertiary
Type 1. Br. TC 11 A	6.0	6.0	
B	5.5	6.0	
C	5.8	6.0	
D	5.5	6.0	5.5
2. Y-SK TC 21 A	6.0	5.5	
B	5.5	6.0	
C	6.3	5.5	6.0
D	5.8	6.0	
3. Leon TC 58	6.0		5.5

is the antitryptic factor which is used to prevent lysis of the clot(7). Kidneys from a single monkey can yield 2000 primary cultures and at least 1000 secondary cultures. Such cultures grow readily when held stationary so that roller drums are unnecessary. The production of virus in large quantities, as required for vaccine production, or other purposes, is possible using sheets of epithelial cells of normal monkeys, bathed in protein-free media at the time of virus inoculation. Proteins from the kidney fragment itself or from the plasma clot are less apt to contaminate the virus yield. Although several workers have shown that virus can be grown in suspensions of kidney fragments without added protein, in our hands the epithelial outgrowth not only is more sensitive to smaller amounts of virus, but yields virus of higher titer. Finally, sheets of epithelial cells grown on glass in the absence of plasma by the present technic may offer certain advantages in observing virus-induced plaques(8).

Summary. 1. Monkey kidney epithelial cells can be cultured on glass without a plasma clot. The kidney fragments are induced to adhere to the wall of the test tube by preheating of the tube (45°C), and partial drying after introduction of tissue. These cultures compare well with cultures prepared in plasma clots, and respond as readily to the introduction of poliomyelitis virus. A great advantage is realized in the simplicity of preparation of these cultures, and their incubation in stationary racks. Exclusive of preliminary preparations and subsequent stoppering of the tubes, two trained workers can prepare well over 1000 cultures in 1 hour. About 2000 such cultures can be provided from a single monkey. 2. Bottle cultures can be prepared by the same technic, and have yielded poliomyelitis virus titers of about 10^{-6} per 0.1 ml inoculum. Titers in tubes or bottles reach their maximum in 24 to 72 hours depending upon the dose of virus inoculated. 3. After a culture has grown to satisfactory dimensions, original tissue fragments may be shaken off and transferred to new vessels. The fragments settle and produce comparable outgrowth in a shorter period of time than in the original culture.

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Non-Creatinine Chromogen and Creatinine Clearance in the Rat. (20711)

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A controversy concerning the significance of renal creatinine clearances in the rat has resulted from conflicting observations. Lippman(1) reported that the exogenous creatinine clearance is much greater than the endogenous clearance. Fingl(2) reported that the creatinine/inulin clearance ratio is depressed toward 1 as the plasma creatinine concentration is raised or after administration of para-aminohippurate or probenecid. Martel, Wang, and Gingras(3) have also suggested that creatinine is secreted by the renal tubule of the rat. On the other hand Friedman(4) has reported the identity of creatinine and inulin clearances in the rat. Recently Muntwyler and Griffin(5) performed "true creatinine" clearances (utilizing adsorption on Lloyd's reagent) and state that the endogenous and exogenous clearances of "true creatinine" approximate each other in the rat. The earlier reports mentioned above were all based upon total chromogen determinations.

In the experiments reported here endogenous creatinine determinations were performed upon the serum and urine obtained from 6 male and 6 female rats of the Slonaker-Addis strain, taken from stock, where they had received a diet which contained 17% protein. Creatinine determinations were performed upon serum and urine samples both by the total chromogen method of Bonsnes and Taussky(6) and by the method of Miller and Miller(7) which utilizes adsorption on Lloyd's reagent to obtain a value for "true creatinine". The results are given in Table I.

In spite of the relatively small number of observations, it is clear without statistical analysis that non-creatinine chromogen constitutes about one-third of the total chromogen in rat *serum*, while it constitutes not more than one-tenth of the total chromogen in rat urine. These values are similar to, but less striking than, those obtained in *plasma* and urine by Muntwyler and Griffin, who used Wistar strain rats, which have a common genetic ancestry with those of the Slonaker-Addis strain. Although Muntwyler and Griffin obtained endogenous and exogenous "true creatinine" clearances that approximated each other, neither their values nor ours account completely for the four-fold difference in endogenous and exogenous total chromogen clearances previously reported(1). It is necessary to mention that determinations of the endogenous chromogen, which depend on the Jaffé reaction, are performed at a level which is

TABLE I. Proportion of Non-Creatinine Jaffé Chromogen in Rat Serum and Urine.

	Total chromogen,		"True creatinine,"		Non-creatinine chromogen (% of total chromogen)	
Sex	Serum conc.	Urine conc.	Serum conc.	Urine conc.	Serum	Urine
	mg/100 ml		mg/100 ml			
♂	.655	5.02	.480	5.16	27	–2
	.678	19.11	.457	17.08	33	11
	.742	14.05	.352	12.28	53	13
	.699	11.20	.374	10.20	46	9
	.723	8.20	.417	7.95	42	3
	.588	14.92	.460	12.80	22	14
♀	.588	11.42	.290	11.42	51	0
	.588	10.52	.374	9.01	36	14
	.480	7.12	.226	6.80	53	4
	.655	9.56	.458	8.99	30	5
	.458	10.11	.331	9.50	28	6
	.458	10.99	.417	10.07	9	8

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barely within the sensitivity of those methods which have been used. If a more precise measurement were devised for plasma or serum chromogen levels less than 1.0 mg/100 ml the difference between the reported non-creatinine chromogen proportions might be reconciled.

The presence of non-creatinine chromogen would have a negligible influence upon the creatinine/inulin ratios obtained at high plasma creatinine concentrations. Work which has not been reported previously (because we could not offer a reasonable explanation), but which is based upon 92 animals, resulted in a creatinine/inulin clearance ratio of 1.26 ± .09 within the serum creatinine concentration range of 9.5 to 15.0 mg/100 ml.

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Effects of Cortisone, Ascorbic Acid and Piromen on Phagocytosis in Mice.*† (20712)

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In considering the role of host mechanisms in resistance to experimentally induced infection, attention was drawn to certain compounds which might augment phagocytic activity. In the experiments described, cortisone, ascorbic acid and Piromen administered parenterally in critical amounts have been shown to alter significantly functional activity of mouse phagocytes *in vivo.*

Methods. Animals. Adult albino (*Mus musculus*) mice of mixed sexes were used in all experiments. The weight range was 24 ± 4 g. *Thorium dioxide analysis.* The ThO_2 uptake by mouse spleens after intravenous injection of Thorotrast‡ was determined by the technic described by Gordon and Katsh(1). Details of technic and precision of the analytical procedure with mouse tissues have been described previously(2). *Peritoneal phagocytosis technic.* The intraperitoneal phagocytosis of *Micrococcus pyogenes* var. *aureus* (FDA 209 P) by phagocytic leukocytes was studied. The organism was grown in (Difco) brain heart infusion broth at 37°C for 18 hours. After 4 hours at refrigerator temperature the culture was diluted with broth to contain approximately 4 x 10^9 organisms/ml which gave a turbidity reading of 650 nephelos in a photonephelometer (Coleman, Model 7). Mice (6 for each determination) were injected intraperitoneally with 0.25 ml of this suspension and sacrificed after 2 hours. Several drops of the peritoneal exudate from each mouse were smeared on a slide and stained with Wright's stain. Exactly 200 phagocytic cells were examined on each slide making a total of 1200 cells for each determination. Activity of these cells was estimated by noting the number of cells containing bacteria (active) or no bacteria (nonactive)(3). In each category the values obtained for the 6 animals were added to give one series of numbers for each determination. Substances tested for effect on activity of peritoneal phagocytes were injected subcutaneously 14 and 2 hours before injection of the bacteria. *Maintenance of Klebsiella pneumoniae type A.* The medium and procedure described by Hoogerheide(4,5) was employed. Colonies from freshly incubated plates were seeded into broth and incubated for 7 hours at 37°C. Plate counts of viable organisms were made in (Difco) tryptose phosphate agar. The mouse LD_{50} of *K. pneumoniae* was about 75 viable organisms. Frequent determinations did not reveal any significant change in virulence over an 11-month period. *Drugs.* Cortisone acetate§ was prepared for injection by trituration with a few drops of Tween 80, after which it was suspended in saline. The Piromen|| preparation employed was a nonantigenic polysaccharide prepared from *Pseudomonas aerugenosa.* The ascorbic acid used was the chemically pure compound purchased from the Nutritional Biochemicals Co. *Analysis of data.* In experiments with ThO_2 the significance of difference between treated and control groups was estimated by calculating "t"(6). The significance of differences obtained in peritoneal phagocytosis experiments was determined by calculating "X^2" (6). This method was also employed in analysing the data obtained in the protection experiments.

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† Presented before Soc. Am. Bact., Aug. 10, 1953, San Francisco, Calif.

‡ Thorotrast is a stabilized colloidal solution of ThO_2 (24-26% by vol.), generously supplied by the Heyden Chemical Corp., New York.

Results. Results of the experiment to determine the effect of administration of different amounts of cortisone, ascorbic acid and Piromen on the splenic uptake of ThO_2 by mice are shown in Table I. Cortisone, in the highest dose used (0.1 mg twice daily), caused an increase in ThO_2 uptake which was barely below the .05 level of significance. The uptake in cortisone treated groups was in every case greater than in the control group, and the uptake increased with administration of increasing amounts of cortisone.

Results with Piromen indicate a critical dose-response relationship for uptake of ThO_2; of the 4 doses of this agent used, ranging from 0.01 µg to 10 µg per injection, only the 0.1 µg dose yielded a significant effect on uptake. By contrast, it is seen in Table I that ascorbic acid in doses of 1 mg and 0.1 mg every 12 hours did not significantly affect the splenic uptake of ThO_2.

TABLE I. Effect of Cortisone, Ascorbic Acid, and Piromen on Splenic Uptake of ThO_2 in Mice.

Treatment and dose/12 hr		mg ThO_2/ spleen	Comparisons to control P
Saline		3.63±.157*	—
Cortisone	.1 mg	4.04±.078	.05+
	.01	3.78±.076	>.1
	.001	3.66±.212	>.1
Ascorbic acid	1	3.29±.225	>.1
	.1	3.36±.190	>.1
Piromen	10 µg	3.76±.664	>.1
	1	3.79±.386	>.1
	.1	4.19±.108	*.05*
	.01	3.30±.366	>.1

* Mean and stand. error.

TABLE II. Effect of Cortisone, Ascorbic Acid and Piromen on Phagocytic Activity of Leukocytes in Peritoneal Cavities of Mice.

Treatment and dose/12 hr		No. of phagocytes Active	Non-active	Comparisons to control P
Saline		256	944	—
Cortisone	.1 mg	261	939	>.1
	1	440	760	*<.001*
Ascorbic acid	1	299	901	*<.05*
Piromen	.1 µg	320	880	*<.01*
	1	239	961	>.1

Table II shows the effect of cortisone, ascorbic acid and Piromen on the phagocytosis of *M. aureus* in the peritoneal cavities of mice. The probability (P) values are based upon the relative numbers of phagocytes in the active or non-active categories in the test groups as compared to the control group. Cortisone in a dose of 1 mg every 12 hours caused a highly significant increase in the number of active phagocytes. Piromen (0.1 µg) also produced a significant increase in phagocytosis, while the higher doses of this agent (1 and 10 µg) were without significant effect. The effective dose of Piromen in this experiment was the same as that which augmented splenic macrophage activity further suggesting a critical dose-response relationship for the action of this agent upon phagocytes. Ascorbic acid (1 mg) caused a slight, but significant increase in phagocytic activity.

Table III summarizes results in a number

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§ Generously supplied by Merck and Co.

|| Generously supplied by Travenol Laboratories, Morton Grove, Ill.

TABLE III. Effect of Cortisone and Piromen on Experimental *Klebsiella pneumoniae* Infections in Mice.

Challenge dose and antibiotic treatment	Treatment and dose/12 hr	Mortality (5 days)	Comparisons to control P
5 LD_{50}	Saline	24/40*	—
	Piromen .1 μg	23/40	$>.5$
	Cortisone .1 mg	34/40	$>.5$
50 LD_{50}; 1 mg chloramphenicol	Saline	29/40	—
	Piromen .1 μg	28/40	$>.5$
	Cortisone .1 mg	31/40	$>.5$
	Piromen .1 μg + Cortisone .1 mg	34/40	$>.1$
10000 LD_{50}; 100 μg aureomycin	Saline	12/40	—
	Piromen .1 μg	9/39	$>.5$
	Cortisone .1 mg	8/40	$>.1$

* Dead/total animals.

of experiments carried out to determine the value in experimental infection of the critical amounts of these agents which apparently potentiated phagocytic activity *in vivo*. In the first experiment groups of mice received subcutaneous injections of these agents 6 hours before intraperitoneal challenge with 5 LD_{50} of *K. pneumoniae* and thereafter at 12-hour intervals throughout the 126 hour observation period. Neither Piromen nor cortisone decreased the mortality of the infected animals.

In the succeeding experiment a modified design was employed; the phagocytosis potentiating agents were tested singly and in combination in mice challenged with 50 LD_{50} of *K. pneumoniae* and treated intraperitoneally one hour later with 1 mg of chloramphenicol. Neither cortisone, Piromen nor the combination of the two drugs produced a significant change in mortality. A third experiment involved the use of chlortetracycline (Aureomycin) in a similar experimental design. Aureomycin could be used in a smaller subcurative concentration than chloramphenicol because of its greater effect on the mouse infection. It is seen that no increased protection was obtained.

In each of the challenge-treated experiments described, the cumulative survival time of the mice in each of the test groups was compared to the control group. In no case was any significant difference noted.

Discussion. A number of workers(7,3,8) have directed their attention to the specific problem of finding agents which augment phagocytic function. Since no term has been proposed for such agents, it is suggested that the word *auxophagocytic* (Gr. *auxo* = to increase) be employed to indicate agents which specifically augment phagocytic activity by their direct or indirect action on phagocytes. The antonymic structure would be *meiophagocytic* and would indicate agents which depress phagocytic action.

It appears from the results presented that in certain dose ranges cortisone, ascorbic acid and Piromen may be auxophagocytic in mice. The agents varied in effect not only with dose but also with site of action, *e.g.*, ascorbic acid did not significantly affect ThO_2 uptake by splenic macrophages but augmented peritoneal phagocytosis. The auxophagocytic action of cortisone may help to explain some of the seemingly contradictory reports concerning the efficacy of cortisone in treating microbial infections.

Ascorbic acid was observed to cause a slight increase in the phagocytic activity of leukocytes. Nungester and Ames(9) showed that the leukocytes from scorbutic guinea pigs were much less active than those from normal animals. Merchant(10) noted a direct correlation between the phagocytic capacity of leukocytes suspended in serum and the concentration of ascorbic acid in the serum until the normal ascorbic acid concentration was reached. The effect of ascorbic acid noted in the present investigation was small and would not appear as a significant increase unless a large number of leukocytes were observed.

Piromen has been shown here to increase the phagocytic activity of both fixed macrophages and leukocytes. While no beneficial effect was noted in mice infected with *K. pneumoniae* the ability of this drug to increase phagocytic activity *in vivo* suggests that it should be evaluated under a variety of experimental conditions.

Summary. Cortisone, ascorbic acid and Piromen were tested in mice for their effect upon macrophage activity as determined from the splenic uptake of colloidal ThO_2 and upon the phagocytic activity of leukocytes in the peritoneal cavity against *Micrococcus aureus*. Cortisone in high doses (0.1 to 1 mg every 12 hours) significantly enhanced phagocytic activity of both macrophages and leukocytes. Ascorbic acid in doses as high as 1 mg every 12 hours had no effect on the activity of macrophages but this dose did significantly enhance the phagocytic ability of leukocytes in the peritoneal cavity. Piromen (0.1 μg every 12 hours) significantly increased the phagocytic activity of both types of cells. However, doses above or below this value were without significant effect in either type of experiment. The more potent auxophagocytic agents (cortisone and Piromen) were without salutary effect when tested singly and in combination in protection experiments in mice challenged with predetermined doses of *Klebsiella pneumoniae*.

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Differentiation of Streptomycin and Dihydrostreptomycin Inhibition, by Means of Reversing Metabolites.* (20713)

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The close chemical relation between streptomycin (SM) and dihydrostreptomycin (DSM) together with the close resemblance of their antimicrobial spectra with respect both to wild type organisms and to strains developed for resistance to each respectively, has led to the widespread current belief that these antibiotics inhibit bacteria by identical mechanisms. The activity of DSM has even been postulated to result from its oxidation to SM by the bacteria(1).

SM and DSM have been extremely difficult to distinguish microbiologically, if indeed it has, with assurance, been possible at all, and frequently the two have been regarded as identical in antimicrobial growth effects(2-6) and in enzymatic effects(7), as well as in dependency experiments(8). Also SM and DSM appear to be absorbed and excreted by the mouse in the same way(6).

On the other hand, there is some evidence suggesting that SM and DSM may not be identical in their antibacterial action. This evidence rests principally on small differences in relative amounts of the two antibiotics required for inhibition of a number of different organisms tested in parallel(6,9). Of interest in this connection is the fact that the neurotoxic threshold dose (especially for the eighth nerve) of DSM in animals is distinctly less than for SM(10).

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TABLE I. Selective Reversal, by Individual Metabolites, of *Aerobacter aerogenes* Growth Inhibition Caused by Streptomycin and by Dihydrostreptomycin. Plates incubated for 34 hr at 37°C.

Reversing agent, μg/ml agar medium	No antibiotic	Antibiotic and μg/ml agar medium: Streptomycin						Dihydrostreptomycin					
		.5	1	2	4	8	16	.5	1	2	4	8	16
None	+	+	—	—	—	—	—	+	—	—	—	—	—
Guanylic acid, 5	+	+	+	+	+	+	—	+	—	—	—	—	—
Cytosine, 5	+	+	+	+	+	+	—	+	—	—	—	—	—
Xanthosine, 5	+	+	+	+	+	+	—	+	—	—	—	—	—
Guanosine, 5	+	+	+	+	+	+	—	+	—	—	—	—	—
Thymine, 5	+	+	+	+	+	—	—	+	—	—	—	—	—
L-phenylalanine, 25	+	+	—	—	—	—	—	+	+	+	+	—	—
L-phenylalanine, 50	+	+	—	—	—	—	—	+	+	+	+	+	—
Yeast autolysate, 1000	+	+	+	+	+	+	—	+	+	—	—	—	—

+ = Growth of approximately 100 discrete colonies.
— = No colonies visible to the naked eye.

This paper reports a qualitative distinction between SM and DSM with respect to reversibility, by selected known metabolites, of the growth inhibition caused by these antibiotics. As used here, the term "reversal" simply refers to the prevention or counteraction of the growth inhibition (irrespective of mechanism) otherwise caused by a given concentration of an antibiotic.

Materials and methods. The spread plate procedure, employing *Aerobacter aerogenes* strain P and M-9 agar was used for screening various known metabolites for their ability to reverse or neutralize otherwise inhibitory concentrations of the particular antibiotics. Composition of M-9 agar, grams per liter of distilled water: dextrose 4.0, NH_4Cl 1.0, $MgSO_4 \cdot 7\ H_2O$ 0.12, Na_2HPO_4 6.0, KH_2PO_4 3.0, agar 15.0. Antibiotics and reversing agents were added as aqueous solutions (0.5 to 1.0 ml) to sterile petri dishes and 10 ml cooled melted agar added. The supplements were mixed thoroughly with the agar by rotating the plates. The agar was then allowed to harden. The plates were spread with 0.05 ml of an aqueous suspension containing approximately 100 washed cells. The procedures were exactly like those described previously(11,12). The term "reversal factor" connotes the ratio of amount of antibiotic required to inhibit in the presence of a reversing agent to that required in the absence of the agent. Colonies in plates containing antibiotic and reversing agent become visible about 4 to 6 hours later than appearance of visible colonies in the basal medium. After the delay, rate of growth of the "reversed" colonies approximates that of the controls, and in about 24 to 30 hours colonies in both are about the same size.

Results. Differential reversal of growth inhibition caused by SM and by DSM. Of fifty metabolites tested in preliminary screening experiments(11,12) only L-phenylalanine and a group of purine and pyrimidine derivatives were found to possess reversing activity against SM and DSM inhibition of *A. aerogenes.* Table I presents the particular reversing compounds, the degree of reversal, and their specificity with respect to SM and DSM. For example, in the absence of reversing agents, 1.0 μg of SM and DSM respectively sufficed for complete inhibition. However, in the presence of the purine and pyrimidine derivatives 16 μg SM was required for inhibition. The presence of these derivatives did not, however, alter the amount of DSM required for inhibition, namely 1 μg, the same as in the absence of the derivatives. Similarly, phenylalanine did not influence the inhibitory level of SM, it being 1 μg irrespective of the presence or absence of this amino acid. However, phenylalanine did increase markedly the amount of DSM required for inhibition as compared to in its absence, *e.g.*, 16 μg vs. 1 μg.

It is evident that guanylic acid, cytosine, xanthosine, guanosine and thymine each displayed reversal of SM inhibition, with reversal factors of 16 for all except thymine and that these compounds displayed no detectable reversal of DSM tested simultaneously. Conversely, L-phenylalanine reversed only DSM inhibition (reversal factor = 16) and had no activity in reversing SM inhibition. The reversing activity of the amino acid was proportional to the concentration used, within the limited range tested. A concentration of 100 μg per ml agar was toxic in the growth control plates. Also, a commercial yeast autoylsate (Basamine-Busch) was 8-fold more effective in reversing SM than DSM. In other experiments, varying concentrations of the reversing agents did not alter the qualitative specificity of the results.

Discussion. The evidence here, together with the absence of cross-reversal with SM- and DSM-reversing substances produced by homologous resistant strains described elsewhere(13), while not conclusively establishing different mechanisms of action of SM and DSM, does contradict the widespread assumption that both act in identical fashion. The reversing compounds may well be related to the biochemical mechanisms interrupted when the cells are inhibited by threshold concentrations of the antibiotics.

Summary. Several purine and pyrimidine derivatives reversed the growth inhibition of *Aerobacter aerogenes* caused by streptomycin under specified conditions. Inhibition by dihydrostreptomycin was not reversed under the same conditions. L-phenylalanine reversed the inhibition caused by dihydrostreptomycin and not that caused by streptomycin.

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Isolation of a Cytopathogenic Agent from Human Adenoids Undergoing Spontaneous Degeneration in Tissue Culture. (20714)

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During the course of a study of the growth of human adenoid tissue in roller tube culture, a characteristic degeneration has been encountered which has been found to be serially transmissible in other tissue cultures.

Methods. Adenoids were obtained during the winter and spring of 1952-53 from operations on young children.† The tissues were minced, washed 3 times in nutrient media, and cultured by the chicken plasma clot technic. The majority of adenoids and all other tissues were grown in a modification of the beef amniotic fluid medium described by Enders (1);‡ a few adenoids were cultured in media that were modified from those of Youngner *et al.*(2).§ Explant cultures of other tissues were prepared in the same manner; HeLa cell cultures(3) were obtained from Microbiological Associates, Bethesda, Md. Culture fluids were changed twice weekly, and the supernatant fluids were stored in screw-capped vials at –20°C. Passages were made by transfer of supernatant fluid in a dilution of 10^{-1}.

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* Laboratory of Infectious Diseases and the Laboratory of Clinical Investigations.

† We are glad to acknowledge the cooperation of Dr. E. Clarence Rice, pathologist, Children's Hospital, Washington, D. C., the Research Foundation, Children's Hospital, Washington, D. C., and the U. S. Naval Hospital, Bethesda, Md., in supplying the adenoids.

Results. During the first week of culture most adenoid cultures demonstrated sheets of epithelium, often ciliated, with a few areas of fibroblastic outgrowth. After 8 to 28 days in culture, 33 of the 53 (62%) adenoids observed for this period demonstrated a characteristic rounding of the peripheral epithelium, progressing to complete destruction of the epithelium within 7 to 10 days. The morphological appearance of the round cells was distinctive, and the appearance of a few of these cells almost invariably was followed by progressive replacement of the epithelium by the same type of cell. These cells were large, round or slightly ovoid, with a smooth, distinct margin, clear peripheral cytoplasm, and a densely granular center, the nucleus obscured by the granulation.

Culture fluids of 14 adenoids demonstrating this degeneration have been transferred to fresh cultures of adenoids, human embryonic tissue, or HeLa cells, and in 13 instances characteristic changes were produced in the recipient cultures. The changes observed in the recipient adenoid or embryonic tissues were identical with the picture of the original degeneration of the adenoids. The changes in HeLa cell cultures consisted of clumping and rounding of the cells, followed by dislodgement of the clumps from the wall of the tube. One isolation (strain No. 2) of the agent was obtained from an adenoid which degenerated on the eighth day of culture, by passage of the supernatant fluid to cultures of a second adenoid, the controls of which did not undergo spontaneous degeneration during 69 days of observation and from which no cytopathogenic agent could be isolated. This strain has been carried through a total of 17 passages, the first in adenoid, one in human embryo trachea, and the last 15 in HeLa cells, with production of the typical changes in every passage. Degeneration of the seventeenth passage tissue occurred one day after infection with fluid representing a theoretical dilution of the original adenoid fluid of 10^{-32}. Fluid of the eleventh passage when passed to cultures of human fetal trachea reproduced the typical round cell degeneration after 3 days. Similarly, other strains of the agent have been carried without difficulty through serial passages, and have consistently reproduced the degeneration. In both human embryo skin and HeLa cell cultures the agent has been found to reach a titer in the supernatant fluid of more than 10^5 infectious units per ml when titrated in HeLa cells.

The incubation period of the cytopathogenic effects in human epithelium is usually 4 to 8 days; with higher dilutions of the inoculum the incubation period may be as long as 23 days. In cultures consisting almost entirely of human embryo fibroblasts the incubation time is longer, ranging from 8 to 15 days for the lowest dilution, to as long as 40 days with higher dilutions. Incubation time in HeLa cells varies from 2 to 20 days, depending on the concentration of the agent. The following tissues have been found to show cytopathogenic effects after infection with the agent: human adenoid; human embryonic nose, pharynx, palate, tongue, trachea, skin, muscle, and pancreas; new-born human prepuce; HeLa cells; suckling rabbit kidney and trachea; suckling hamster trachea, lung, kidney, skin, and muscle; and chick embryo lung and skin. The effects seen in hamster and chick embryo

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‡ The medium consisted of beef amniotic fluid 85%, beef embryo extract 10% and inactivated horse serum 5%.

§ During the first week of culture the following medium was used: Hanks' solution 40%, inactivated horse serum 40%, and chick embryo extract 20%. After this time the medium was changed to the following: Hanks'-Sims' solution (3:1) 85%, inactivated horse serum 5%, and chick embryo extract 10%. All media contained phenol red (0.001%), penicillin and streptomycin (50 to 250 units of each), and soybean trypsin inhibitor (0.005%).

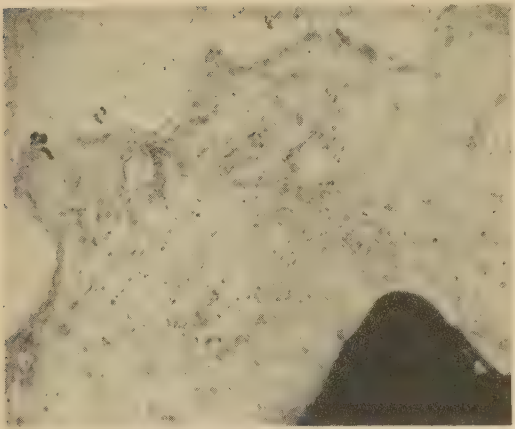

FIG. 1. Epithelial sheet of uninoculated human fetal trachea, 48th day of culture. H & E stain.

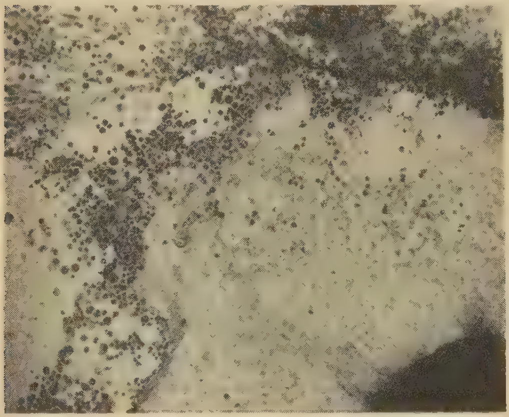

FIG. 2. Human fetal trachea, 48th day of culture, 19 days after infection with A.D. agent, strain #2. H & E stain. ×37.

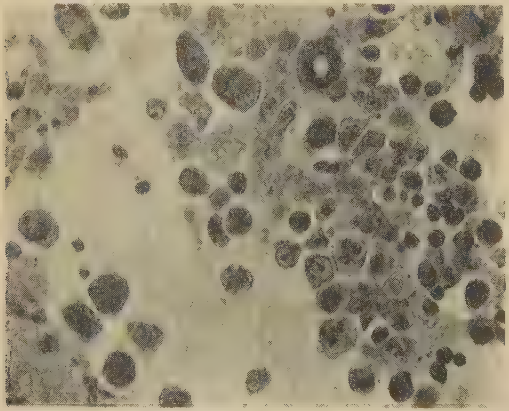

FIG. 3. Higher power photomicrograph of tissue shown in Fig. 2. H & E stain. ×146.

cultures were not distinctive, being characterized by gradual death and disappearance of tissue with lysis of the plasma clot. The following tissues have shown no evidence of cytopathogenic changes following inoculation with the agents: beef embryonic trachea, mouse embryo mixture, mouse glioblastoma, monkey testis, and guinea pig trachea and kidney.

Hematoxylin-eosin stained preparations of infected human embryonic tracheal epithelium cultures have been examined by Dr. Henry Pinkerton, St. Louis University School of Medicine, who made the following description: "The normal cells take the hematoxylin stain, both nucleus and cytoplasm. The affected cells show *eosinophilic* swollen nuclei, with what I take to be nuclear inclusions. These are usually basophilic, but occasionally eosinophilic. There is margination and fragmentation of the nuclear chromatin (which stains deeply blue-black), often wrinkling of the nuclear membrane, and swelling of the nuclei. The cytoplasm stains deep reddish purple, and occasionally contains 10 to 12 small pale blue inclusions'. . . . Often, also, the nucleus is blended with the cytoplasm to form a granular purplish mass in which no nucleus can be recognized". Photomicrographs of stained roller tube cultures are shown in Fig. 1-3.

The agent has not been found to grow on bacteriological media, including repeated cultures on thioglycollate broth, blood agar, and several types of pleuropneumonia media.‖ Activity of the agent was destroyed by heating at 62°C for 30 minutes; the agent was filterable through a Mandler No. 14 candle with some loss in titer. No loss of activity was found after storage at 3°C for a week or after three cycles of quick-freezing and thawing. No clinically recognizable disease has been produced by the agent inoculated by various routes into experimental animals, including embryonated eggs, suckling and adult mice, suckling hamsters, guinea pigs, rabbits, rhesus monkeys, and a chimpanzee.

Hyperimmunization of rabbits with agent-containing tissue culture fluids resulted in the production of antibodies capable of neutralizing the agent in tissue culture, whereas immunization with control culture fluid did not.

‖ We are indebted to Drs. Ruth Wickelhausen and Ella Verder for the attempts to isolate pleuropneumonia-like organisms.

Rabbit antisera against herpes simplex and vaccinia viruses did not neutralize the agent in tissue culture, and rabbit hyperimmune serum against strain No. 2 of the adenoid agent did not neutralize herpes simplex virus in suckling mice or vaccinia virus in tissue culture. The cytopathogenic effects of the agent were prevented or significantly delayed by addition to the cultures of human gamma globulin in a final concentration of 1:500 or some human sera in dilutions of 1:50 or higher, whereas other human sera showed no neutralizing capacity at dilutions of 1:25.

In this laboratory a variety of human viruses have been inoculated into human adenoid and embryo cultures and HeLa cell cultures, including herpes simplex, vaccinia, influenza A′ and B, Type 1 poliomyelitis, and members of the Coxsackie A and B groups, as well as human pleuropneumonia-like organisms and presumably virus-containing materials from cases of measles and varicella; in no instance has the picture produced by the adenoid agents been produced. The pattern of destruction of adenoid epithelium by herpes simplex and poliomyelitis viruses was somewhat similar to that of the adenoid agents, but their effects on HeLa cells did not resemble that produced by the adenoid agents.

Summary. 1. From the present evidence it appears that an unidentified, possibly new, tissue culture cytopathogenic agent has been isolated repeatedly from human adenoids undergoing spontaneous degeneration in tissue culture. The filterability and the inability to cultivate the agent on bacteriological media and to demonstrate organisms in stained tissue culture preparations would indicate that the agent belongs to the group of viruses or rickettsiae. It is tentatively proposed to designate the agent as the "adenoid degeneration agent", abbreviated as "A.D. agent". 2. That the agent is derived from the adenoid tissue rather than from the nutrient media is indicated by the fact that some adenoids and all human embryonic tissues cultivated in the identical media and at the same time have not undergone degeneration, although they are susceptible to infection with the agent; also, repeated attempts to isolate the agents from adenoid cultures not demonstrating degeneration have been uniformly unsuccessful. 3. Further investigation is in progress to determine the relation of the agent to the adenoids and to study their possible role in human disease; particularly upper respiratory infections.

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Effect of Buffer on Paper Electrophoretic Studies on State of Yttrium in Blood.* (20715)

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When an inorganic compound is injected into an animal its physico-chemical state may be altered by the blood, which is a highly complex fluid containing various inorganic ions and organic compounds, especially proteins. Substances introduced into blood may be complexed and thereby kept in solution or may aggregate to form colloids which are largely removed by the reticulo-endothelial system. To investigate the physico-chemical state of certain radioactive tracers in the blood stream, we have used filter-paper electrophoresis with

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† Oak Ridge Institute of Nuclear Studies Research Participant from Division of Physiology, University of Tennessee, Memphis.

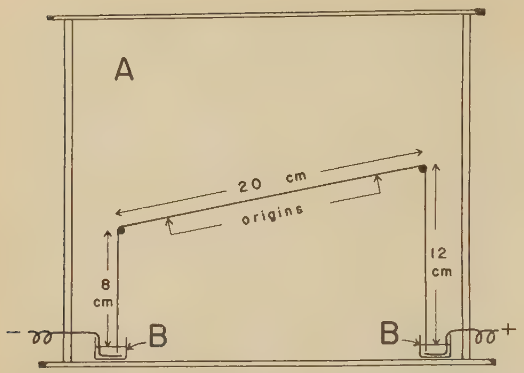

FIG. 1. Paper electrophoresis apparatus. A is a covered glass cylinder. B are cups or troughs of buffer solution containing carbon or nichrome electrodes. Paper strips supported on 2 glass rods.

a modified buffer system. In the electrical separation of plasma proteins, most investigators have used barbiturate (veronal) buffer of pH 8.5, but the possible effects of the buffer upon labeled plasma were not investigated. To determine whether results obtained by this technic are truly representative of the conditions in blood, we used radioactive yttrium as a test material to study the effects of different buffers upon the electrophoretic patterns.

Methods. The yttrium-91 used was carrier-free and therefore present in a low enough concentration so as not to affect the system under study. The chemical behavior of Y^{91} in pure solutions has been studied by Kurbatov and Kurbatov(1) and Schweitzer and Jackson (2) who found that at a pH above 3, a portion of the yttrium will no longer pass through filter paper and becomes partially removable by centrifugation. Such behavior is characteristic of radiocolloids. Thus, it is probable that yttrium at the pH of blood plasma exists, at least in part, in colloidal form unless one or more of the normal blood constituents prevent colloid formation by their complexing action.

The apparatus, Fig. 1, was essentially that of Gordon *et al.*(3). Evaporation of water from the strips was reduced, though not entirely prevented, by covering the walls of the outer container with moist filter paper. Filter paper strips were moistened with various buffers, then 5-10 μl of sample containing 0.1 μc of Y^{91} was applied in a narrow band at a marked position on the strip. A voltage gradient of 5 volts per cm was maintained during the experiment.

First we followed the movement of a pure solution of radioactive yttrium chloride dissolved in 0.05 N HCl and applied to individual paper strips A, B, and C in Fig. 2, buffered respectively with 0.05 N HCl, 0.2 M KH phthalate (pH 4), and 0.05 molar sodium barbital-barbituric acid (pH 8.5). The autoradiograms show that after 1 hour the yttrium had migrated only in the acid solutions, and the phthalate band was not sharp. There was no movement in the barbital buffer.

Next, sheep plasma was incubated *in vitro* 30 minutes with Y^{91} chloride, and an aliquot placed on strip D (Fig. 2), which was dipped into barbiturate buffer. As with strip C, no migration occurred. It is evident that yttrium in plasma and yttrium chloride behave similarly when the separations are carried out in the standard barbiturate buffer.

In an attempt to avoid possible alterations of the physico-chemical state of plasma yttrium, we then substituted heparinized sheep plasma for the barbiturate buffer, strip E. Labeled plasma from the same animal that supplied the buffer plasma was placed at the origin of this strip just as for strip D. Now a fraction of the plasma did migrate, forming a distinct band containing approximately 30% of the Y^{91} (as determined by mica-window GM tube counting). Evidently yttrium in plasma existed in at least 2 fractions, and the mobile fraction did not appear when electrophoresis was carried out in barbiturate buffer.

Discussion. Schulz and Wegner(4) pointed out that the migration of materials in filter paper electrophoresis is determined by 3 factors: by electromigration, by the movement of solvent on the strip caused by evaporation and electroendosmosis, and by interaction of some materials with paper. To determine whether the observed movements of yttrium were due primarily to solvent flow or to electrophoresis, applications of Y^{91} were made to 3 different locations on each of strips F and G, which were buffered in 0.05 N HCl and sheep plasma, respectively. After an hour, the mobile fractions of the endspots migrated toward the center

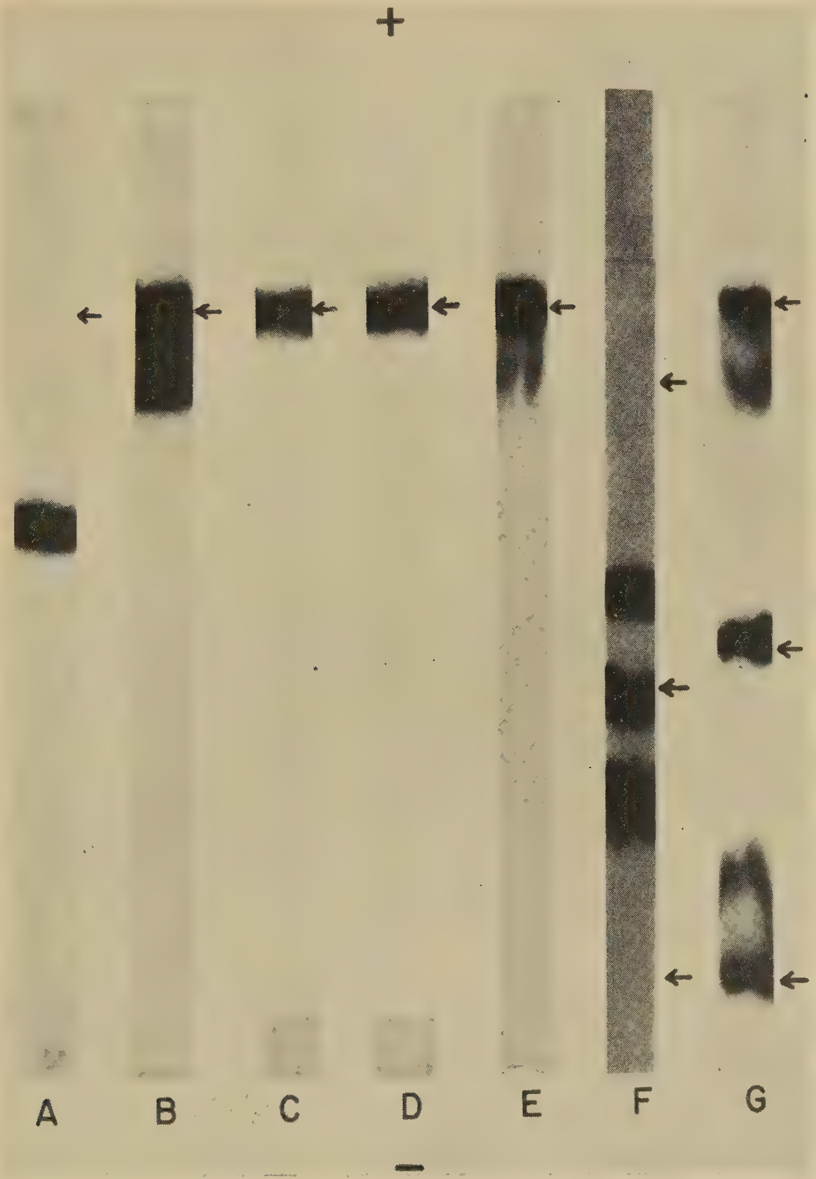

FIG. 2. Autoradiograms of paper strips. Arrows indicate points of application of samples.

while the center spot did not move. Such behavior would be characteristic of an uncharged or very slightly charged molecule which was simply washed along by the solvent, which flows toward the middle of the paper strip(4). Using the same technic with $Ca^{45}Cl_2$ we observed that the radioactive calcium always migrated toward the cathode, in plasma as well as in HCl buffers, though the rate of migration depended on the site of sample application. Thus, even though the movement of materials on paper strips is not necessarily the simple result of electromigration, the additional flow of solvent may be useful in the differentiation of ionic and neutral molecules.

Summary. The use of blood plasma as a buffer for filter paper electrophoresis makes it possible to study the physico-chemical state of injected materials or of native blood constituents without subjecting the blood fractions

to possible alterations by an artificial buffer. In veronal buffer yttrium appeared to exist in wholly immobile forms. In plasma buffer, yttrium existed in an immobile fraction and in an unionized fraction which moved with the solvent. Migration of materials as a result of solvent flow must be considered as a factor in the interpretation of electrophoretic studies of this type.

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Effect of 2,3-Dimercaptopropanol (BAL) upon Distribution and Excretion of Plutonium.* (20716)

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Introduction. The transuranic element, plutonium, presents a major health hazard due to its very toxic alpha radiation, long half life, high degree of retention by the skeleton, and extremely slow rate of elimination from the body(1). Although the sulfhydryl compound, 2,3-dimercaptopropanol (BAL) has been shown to have no significant influence on acute poisoning of animals with uranium salts(2) and strontium^{89}(3), it has proven effective in the treatment of systemic metal poisoning with arsenic(4-6), mercury(7), antimony(8), and polonium(9).

Because of the potential importance of plutonium poisoning, the following study was carried out to determine if therapy with BAL might alter the distribution and increase the excretion of this element.

Experimental. Seventeen young adult female Long-Evans rats ranging in weight from 200 to 240 g were each injected intramuscularly with 15 μg of hexavalent Pu^{239} (half-life 2.43 x 10^4 years) as the chloride into the right front leg. The volume injected in each case was 0.25 ml of physiological saline at pH 3. An amount equivalent to the injected dose was made up to volume in a volumetric flask and aliquots were taken to provide standards.

The animals were divided into 3 groups: 1) Controls. These received no treatment. 2) BAL treated. These animals each received 9 injections of 10 mg BAL in 0.1 ml peanut oil in the muscles of the hind leg, at 4-hour intervals following the injection of plutonium. This dose was well below the LD$_{50}$ of 105 mg/kg reported for intramuscular injection in rats(5), and in the range of effective doses used in treatment of mercury and arsenic poisoning(2,5). 3) BAL-pretreated. These rats received a single injection of 10 mg BAL 5 hours prior to administration of plutonium, in order to ensure a high BAL level in the tissues at the time of injection of the metal. They then received the same treatment as the animals in group 2. Each rat was placed in an individual metabolism cage and fed *ad libitum* on the stock ration. Average weight loss was 5-6 g during the experiment. Urine and feces were collected separately at 2 days and 8 days following injection of plutonium. At the end of 8 days, the rats were sacrificed, and liver, kidneys, right femur, right foreleg and left foreleg were removed, dried and ashed at 650°C for 10 hours. The remainder of the animal or "carcass" and the urine and feces were also dried and ashed. The ashed samples

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TABLE I. Effects of BAL upon Distribution and Excretion of Intramuscularly Injected Plutonium.

	% of administered dose of plutonium*		
Sample	Controls (7 rats)	BAL-treated (5 rats)	BAL-pretreated (5 rats)
Liver	7.1 ± .8†	8.1 ± 1.3	8.0 ± 1.3
Kidneys	.7 ± .1	.8 ± .1	1.3 ± .6
Femur	1.8 ± .1	1.1 ± .3	1.5 ± .1
Carcass	35.6 ± 3.0	31.8 ± 6.5	27.8 ± 2.5
Unabsorbed in inj. leg	43.5 ± 2.6	48.4 ± 5.9	53.0 ± 5.0
Urine 0-2 day	5.5 ± .8	4.7 ± 1.1	3.6 ± .8
Urine 3-8 day	.9 ± .2	.6 ± .1	1.0 ± .2
Feces 0-2 day	1.3 ± .3	.7 ± .3	.7 ± .2
Feces 3-8 day	3.6 ± .6	3.8 ± .8	3.1 ± .4

* Values in this table have been adjusted to 100%. The actual recoveries approximated 89 ± 6% of administered doses. Carcass values have been corrected for plutonium present in right front leg bone as separate from amount retained in injected muscles of that leg.

† Stand. error of the mean: $\text{S.E.} = \sqrt{\dfrac{(x-x')^2}{n(n-1)}}$.

were dissolved in N/10 hydrochloric acid, made up to volume, and the plutonium in a suitable aliquot was assayed by the method of Scott *et al.*(10). The alpha radiation was measured with an ionization chamber and linear amplifier.

Results. The results are shown in Table I. The difference between the plutonium in the right (injected) leg and the left leg gave a measure of proportion of the dose which remained unabsorbed at the injection site. In all three groups, almost half of the administered plutonium remained unabsorbed at the injection site even after 8 days. There was no statistically significant difference in the distribution and excretion of plutonium in the treated groups as compared to the controls. This contrasts with the effect on arsenic, mercury and polonium, and may indicate a lack of reäctivity of plutonium with the sulfhydryl groups of BAL.

Summary. Intramuscular injection of 2,3-dimercaptopropanol (BAL) in doses therapeutically effective in arsenic and mercury poisoning had no effect on the distribution and excretion of injected plutonium (hexavalent), whether given prior to or following the administration of the metal.

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Action of Parathyroid Extract on Bone Phosphorus and Calcium in the Rat.* (20717)

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Recent studies have confirmed the ability of parathyroid extract to produce a marked phosphuria both in the dog(1) and in the rat (2). The ability of the extract to raise serum calcium has also been reemphasized(3,4). However, while Talmage *et al.*(5) reported a slight but significant difference in the rate of uptake of radiocalcium by the bones of normal and parathyroidectomized rats, they were unable to demonstrate by autoradiographs any difference in the distribution of radiocalcium in the bones of normal, parathyroidectomized, or rats administered parathyroid extract. Tweedy *et al.*(6,7) noted some decreased uptake of radiophosphorus in the femurs of rats under parathyroid extract administration as compared to controls. However, localization of these differences to specific sites within the bone was not done.

The following work was undertaken to determine whether the marked phosphuria produced by injection of parathyroid extract was accompanied by any significant changes in the distribution of radiophosphorus and radiocalcium in the long bones of the rat; and to compare calcium and phosphate changes produced by the extract in bone, serum, and urine.

Materials and methods. Young male mature Wistar rats, weighing between 160 and 200 g were used in these experiments. All animals were given intraperitoneal injections of either 5 microcuries of P^{32} or 10 microcuries of Ca^{45}. They were then placed in metabolism cages for urine collection. Parathyroid extract‡ was administered subcutaneously in repeated doses over the experimental period. Urine collections were made at intervals during the experiment and blood was drawn for calcium and phosphate determinations immediately prior to sacrifice. The femurs used for autoradiographs were removed immediately after sacrifice. The following three experiments were run, each consisting of a duplicate series utilizing radiophosphorus (P^{32}) in one, and radiocalcium (Ca^{45}) in the other. *A*. The radioisotope was administered two hours before the injection of parathyroid extract. 500 i.u. of the extract were administered in three equal doses given at hourly intervals. All animals were killed ten hours after the first injection of the extract. *B*. The radioisotope was given 24 hours before the first injection of parathyroid extract. 300 i.u. of the extract were given over a seven hour period and the experiment was terminated 24 hours after the first injection of the extract. *C*. The radioisotope was given 24 hours before the first injection of parathyroid extract. Each experimental animal received 300 i.u. daily for two days (total 600 i.u.) and was killed 48 hours after the first injection of the extract. Radioactivity determinations were made by standard procedures with a Geiger-Mueller counter. Serum calcium determinations were made using Clark-Collip's Modification of the Kramer-Tisdall Method. Urinary and serum phosphate determinations were made by the method of Fiske and Subbarow.

For autoradiographs, the bones were cut longitudinally (medial sagittal section) into approximately equal halves. The bones were then placed in an X-ray cassette and exposed on Kodak, type A X-ray film. The cassettes were placed in presses to insure uniform contact between the bones and the film(8). Since the bones from a given experiment were exposed simultaneously to the same sheet of film, quantitative comparisons were readily obtained. The densitometric measurements were recorded from a Welsh Densichron.

Experimental data. The data from these experiments are tabulated in Table I. The various changes produced by parathyroid ex-

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† Oak Ridge Institute of Nuclear Studies (Research Participant from Rice Institute, Houston, Texas).

‡ Supplied through the courtesy of Eli Lilly and Co., Indianapolis.

TABLE I. Effect in Rats of Parathyroid Extract on Blood Levels, Excretion and Removal from Bone of Calcium and Phosphorus.

Description		Terminal serum values, mg/100 ml		Urinary excretion† of radioisotope		Urinary excretion of PO_4, mg	Optical densities† of bone radioautographs	
		Ca	PO_4	Ca^{45}	P^{32}		Ca^{45}	P^{32}
A. 500 i.u. PTH ext. given 2 hr after isotope dosage, animals sacrificed 10 hr later.	Controls* Exp.*	12.1 14.3	10.2 7.1	100 25	100 2900		100 100	100 84
B. 300 i.u. PTH ext. given 24 hr after isotope dosage, animals sacrificed 24 hr later.	Controls* Exp.*	10.5 14.4	8.3 7.2	100 160	100 4620	.79 17.5	100 87	100 78
C. 600 i.u. PTH ext. given 24 hr after isotope dosage, animals sacrificed 48 hr later.	Controls* Exp.*	11.9 13.6	8.3 12.9	100 220	100 3523	1.68 33.1	100 77	100 41

* 4 rats used for calcium and 4 for phosphorus.

† For ease of comparison, values for control animals have been set at 100 and experimental corrected to this.

tract in bone, serum and urine are summarized below.

Phosphate and P^{32} changes. It may be noted that an immediate and very marked increase in phosphate excretion by the kidneys occurred in all animals receiving hormonal treatment. The average daily excretion of $PO_4^{=}$ by the control rats used in these experiments was approximately 1 mg. The average serum inorganic phosphate value was determined to be about 8.5 mg %. Rats, of the weight used, have a total serum volume of less than 10 ml. Therefore, under these experimental conditions it appears that the control rats excreted daily, through the kidneys, an amount of $PO_4^{=}$ equivalent to the total amount of inorganic phosphate present in the serum. With the addition of parathyroid extract, this excretion increased to between 15 and 25 mg during the first 24 hours of treatment. It is obvious that this amount of phosphate must have come from sources other than inorganic serum phosphate.

Following radiophosphorus administration, the major portion of the radioactivity taken up by the long bones is found to be deposited immediately below the epiphyseal plate, that is in the region of the primary spongiosa. This distribution can be seen as early as one hour after injection of the radiophosphorus and changes thereafter in intensity only, reaching its peak in approximately 24 hours. Following parathyroid extract administration there is a comparatively rapid removal of this radioactivity from the epiphyseal region of the long bones. This loss is significant by 10 hours after the start of parathyroid extract administration (Table I). After 48 hours of treatment, the loss of radiophosphorus from the epiphyseal region is of such magnitude that densitometric measurements show a loss of over 50%, and examination of the radioautographs shows the radioactivity in sections of the epiphyseal region to be reduced to the level of the remainder of the bone (Table I, Plate I). Since the radiophosphorus is diluted considerably with stable phosphorus upon reaching the bone, the removal of the activity indicates the removal from the bone of a considerable amount of phosphate.

When given in sufficient quantity, parathyroid extract will produce in most animals a rise in serum calcium values and a corresponding decrease in serum phosphate levels. This was found to be true here with one important difference. In all determinations made, the serum phosphate value fell during the first 24 hours, being significantly lower by 10 hours. However, as can be seen in Table I, Experiment C, a majority of the animals carried through two days of high level parathyroid extract treatment, showed a terminal serum

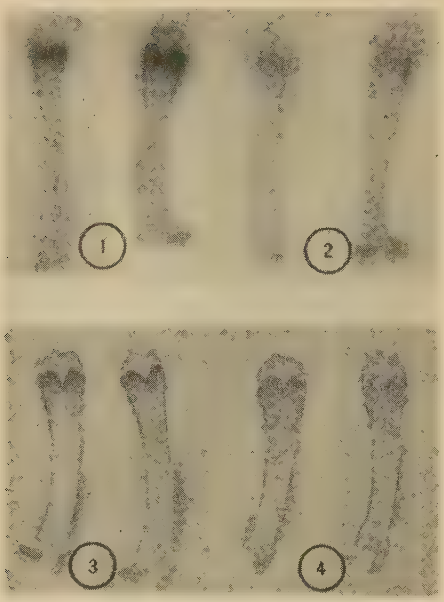

PLATE I. Removal of radiophosphorus and radiocalcium from the femurs of rats by parathyroid extract. Radioactivity given 24 hr before beginning of parathyroid extract administration. Animals killed 48 hr after first inj. of extract.

FIG. 1. Femurs of control rats showing P^{32} distribution.

FIG. 2. Experimental animals showing the marked removal of P^{32} from the subepiphyseal region.

FIG. 3. Control rats showing Ca^{45} distribution.

FIG. 4. Experimental animals showing some removal of Ca^{45} from the subepiphyseal region.

phosphate level which was well above normal (average 12.9 mg %). These animals also showed an increasing antidiuresis and a decreasing output of phosphate in the urine during the second 24-hour period. These effects were probably due to calcium precipitation in the kidney, interfering with its function, a point which will be discussed in more detail below. It is important to note that the output of phosphates during the second 24-hour period was still at least ten times that of the controls, showing that despite the increased (over normal) rate of $PO_4^{=}$ excretion, this ion was entering the serum faster than it was being eliminated, causing an elevation in serum phosphate levels.

Calcium and Ca^{45} changes. Following parathyroid extract administration, radiocalcium excretion values by the kidney dropped over the first 10 hours, followed by a gradual increase, so that at both the 24-hour and 48-hour period, the extract-treated animals showed a slightly increased urinary excretion of Ca^{45} (Table I). This eventual increase in the rate of excretion of calcium by the kidney has been explained in the past as resulting from the elevation of the serum calcium level.

Radiocalcium, once deposited in the bone, was not so easily removed as was the radiophosphorus. The distribution of this radioelement in the bone is essentially the same as that of radiophosphorus. It can be seen from Table I that no evidence of removal was apparent by 10 hours after parathyroid administration, but that by 24 hours there appeared to be sufficient removal to be detected by examination of the autoradiographs. During the second 24 hours, the removal of radiocalcium from the epiphyseal region became more noticeable, but did not begin to compare with the removal of radiophosphorus. The radioautographs of the femurs of animals treated for 48 hours are shown in Plate I, Fig. 3 and 4. During the second 24-hour period calcium was deposited in the kidney cortex. The deposition of calcium was determined on removal of the kidney, both by chemical and radiochemical means. The radioactivity measurements showed an activity in the kidneys of the experimental animals of 365 times that of the controls. Total calcium determinations showed that approximately 12 mg of calcium had been deposited in the kidneys. Similar measurements taken after the first twenty-four hours showed no significant difference between the two groups.

Discussion. It is apparent that parathyroid extract, when given in comparatively large doses is able to remove considerable amounts of calcium and phosphate from the bone. Based on the removal of radioactivity, it would appear that phosphate is removed to a greater extent than is calcium. This is further indicated by the fact that for the first 24 hours almost 20 mg of $PO_4^{=}$ were lost through the kidney, while the corresponding loss of calcium through the kidney or by deposition in the kidney was negligible. However, much of the apparent difference in the movement of these two ions from the bone may possibly be explained by the phenomenon of exchange. If it is supposed that a function of parathyroid extract is to dissolve bone or at least to place these two ions in a chemical condition where they are more readily exchangeable, then the greater removal of radiophosphorus may be due to the fact that there is a large pool of phosphate outside of the bone compared to little or no calcium, allowing much of the radiophosphate to exchange with stable ions. This is further indicated by the fact that the extract increased the excretion of inorganic phosphate by a factor of 20, while the increase in radiophosphate excreted was by a factor of 40. The differences in the increase in urinary excretion of calcium and phosphate may be partially explained by the removal of phosphate from sources other than bone and by possible changes in intestinal excretion not yet studied. However, the possibility that phosphate can be moved in and out of bone at a rate different than that of calcium is a point that cannot be dismissed lightly and deserves further study.

Of prime interest is the bearing of this study on the site of action of the hormone of the parathyroids. Since this study utilized an extract of the parathyroids which admittedly is far from pure, caution must be taken in assuming that all the results attained are normally a function of the parathyroid hormone. However, Kraintz *et al.*(9) have reported a similar study with radiophosphorus using normal and parathyroidectomized rats in which the conditions presented here are essentially reversed, indicating that these results can basically be attributed to the hormone of the parathyroid gland.

With the reservation of possible side effects from the use of a crude extract, the results presented here may be interpreted to show that the parathyroids exert a *direct* effect on bone metabolism as well as an *indirect* effect produced by the increased excretion of phosphate through the kidney. Evidence for this hypothesis is the terminal high serum phosphate values. It would seem that should the effect be on the kidney alone, the serum phosphate level would be expected to remain at normal level or lower as long as the phosphate excretion by the kidney was elevated above normal. In these experiments, during the second day of prolonged high level administration of parathyroid extract, the phosphate excretion was restricted due to a rather severe anti-diuresis probably due to deposition of calcium. The excretion of phosphate was, during this period, still about 10 times normal. The serum phosphate levels, however, rose considerably above normal, indicating that inorganic phosphate was pouring into the serum at a faster rate than it was being excreted. It seems plausible to conclude, therefore, that parathyroid extract directly affects phosphate metabolism both in the kidney and the bone, possibly through a similar enzyme system.

Summary. 1. Effect of parathyroid extract upon removal of calcium and phosphate from the long bones of rats was studied by the use of radiocalcium and radiophosphorus. Autoradiographs showed that significant amounts of both phosphate and calcium could be removed from the subepiphyseal plate region of the long bones of rats by parathyroid extract administration; the amount of radiophosphorus removed was greater than the radiocalcium. 2. These experiments indicate further that parathyroids act directly on bone to produce this removal of calcium and phosphate, and that this effect is enhanced by an additional effect on the kidney whereby the excretion of phosphate is increased. This assumption is based on the fact that serum phosphate values, which are initially reduced by parathyroid extract can be raised above normal by extended treatment of the extract. This increase in serum phosphate levels was accomplished while kidney excretion of phosphate was still approximately ten times the normal daily output, indicating that phosphate was entering the blood at a faster rate than its withdrawal, presumably primarily from the bone.

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Bioassay of Mouse Pituitary Gonadotropin by Induced Ovulation in Pregnant Mice. Evaluation and Biologic Application.* (20718)

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(Introduced by D. W. Fawcett.)

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The induced ovulatory reaction in pregnant mice has been suggested by Burdick and Whitney(1) as a bioassay for gonadotropins. We have recently shown this reaction to be a sensitive assay for gonadotropin content of the mouse pituitary(2), and have used it to show changes in synthesis and release of gonadotropin by the anterior pituitary during gestation(3). This report presents data on the responsiveness of the ovaries of pregnant mice to injections of a commercially standardized preparation of human chorionic gonadotropin. In addition, the differences in the responsiveness of the ovaries to injected homogenates of mouse pituitaries obtained at parturition and at the first postpartum ovulation are shown.

Materials and methods. Swiss female mice, 3 to 9 months of age and approximately 14 to 19 days pregnant, were used for assay animals. The care of animals and the procedure of bioassay of mouse pituitary for gonadotropins has been described earlier(2), so that they need only brief mention here. Commercially standardized human chorionic gonadotropin (HCG)§ was employed for the determination of the responsiveness of the pregnant animals' ovaries. The gonadotropin was made up in the desired concentrations in physiological saline solution and injected intraperitoneally in a uniform volume. Pituitary glands to be assayed were collected from animals which had been used in a previous study to determine the relationship between the time of parturition and ovulation(4). These glands were dehydrated in acetone, desiccated in vacuum and stored. Preparatory to assay they were homogenized, suspended in saline and serially diluted. The homogenates were injected intraperitoneally. The frequency with which ovulation was observed in groups of pregnant assay animals was adjusted by the statistical procedure recommended by Reed and Muench(5). This technic has the effect of augmenting the number of responses at the dosages on either side of the 50% end point. Since this design partially offsets chance variation, the method defines the end point more precisely than would otherwise be possible.

Results. The ovulatory responses of 144 pregnant female mice which received HCG in varying amounts are summarized in Table I. The dose of HCG that would have been required to produce a response in 50% of the animals has been interpolated from the adjusted values. This calculated 50% end

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§ We are indebted to Drs. A. P. Grieve and A. S. Cook of Ayerst, McKenna and Harrison for generous supplies of human chorionic hormone.

TABLE I. Ovulatory Responses of Pregnant Mice to Injections of Human Chorionic Gonadotropin.

Dosage, I.U.	No. of pregnant mice	No. induced to ovulate	% ovulation observed	% ovulation adjusted*	Total No. of ova	Avg No. ova/assay animal
.125	31	2	6.5	2.2	3	.10
.25	33	3	9.1	7.6	18	.55
.50	26	11	42.3	38.6	70	2.69
1.0	27	21	77.8	80.1	140	5.19
2.0	27	24	88.9	95.2	215	7.96

* Method of Reed and Muench(5).

TABLE II. Ovulatory Responses of Pregnant Mice to Mouse Pituitary Glands Obtained at Time of Parturition and at First Postpartum Ovulation.

Time of pituitary removal	Dosage in pituitary equivalents	No. of pregnant mice	No. induced to ovulate	% ovulation observed	% ovulation adjusted*
Parturition	1/8	12	1	8.3	2.9
	1/4	17	3	17.6	13.1
	1/2	19	9	47.4	41.7
	1	13	8	61.5	65.1
	2	10	7	70.0	86.5
Postpartum ovulation	1/2	13	1	7.7	2.9
	1	12	1	8.3	8.4
	2	21	9	42.9	42.0
	4	10	8	80.0	87.3

* Method of Reed and Muench(5).

point is 0.60 I.U. and agrees reasonably well with the 0.75 to 1.0 I.U. obtained by Burdick *et al.*(6) in diestrous mice.

Data on the responses of 127 assay animals which received varying equivalents of pituitary glands are presented in Table II. Homogenates of pituitary glands collected at the time of parturition indicated the 50% end point lying between ½ and one pituitary. The end point was calculated to be at the level of 0.58 pituitary equivalent. Pituitaries obtained at the time of the first postpartum ovulation showed the 50% response to be located between 2 and 4 pituitaries. The end point was calculated to be at the level of 2.25 pituitary equivalents. The difference between these end points indicates that almost a 4-fold decrease in the gonadotropic potency of the pituitary gland was detected during the 15-hour period between parturition and the first postpartum ovulation.

Discussion. The present experiments provide further demonstration that the induced ovulatory response of pregnant mice is an immediate and sensitive indicator of the potency of both human chorionic and mouse pituitary gonadotropins. This response has been separated into (a) a qualitative estimate based upon the proportion of animals ovulating at a given dosage level and (b) a quantitative evaluation based upon the number of ova extruded from the ovaries at any given dosage level (Table I). Application of the quantitative response has been reported previously(3). Our present report applies the qualitative response in order to determine changes in the gonadotropic potency of the mouse pituitary gland between parturition and the postpartum ovulation (Table II).

Advantages of establishing dosages at which half the animals will respond have been summarized by Gaddum(7). This end point is less affected by chance variations than is any other qualitative end point. The frequencies of ovulation are given in Tables I and II and are graphed in Fig. 1. The steepness of these curves both for the standard preparation and for the 2 types of pituitary glands indicates the sensitivity of the assay animals. In addition, the slopes of the 3 curves are comparable thereby attesting to the uniformity in response of the assay animals. An assay procedure

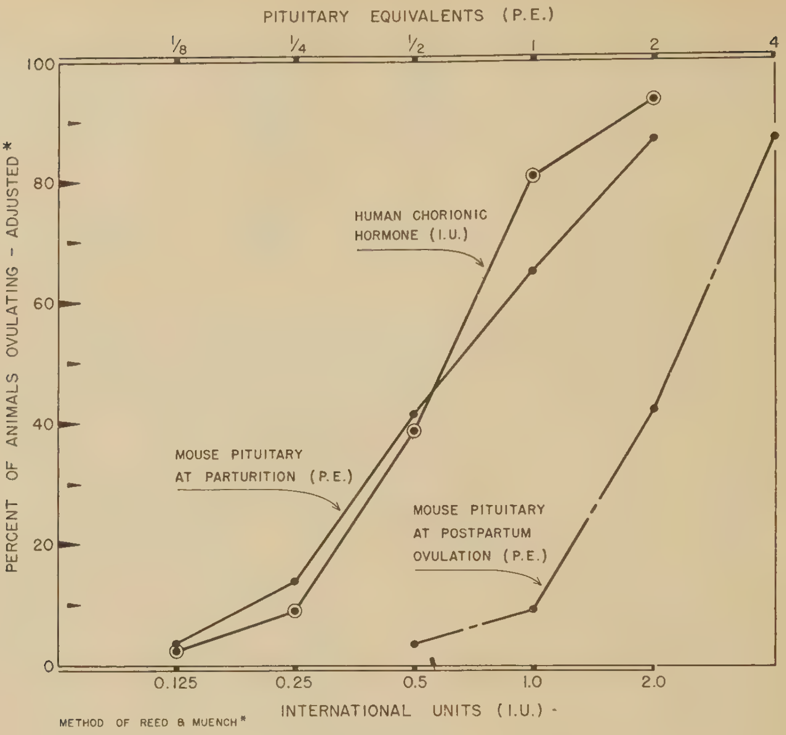

FIG. 1.
Ovulatory responses of pregnant mice to human chorionic and mouse pituitary gonadotropin.

having a steep slope and a reproducible dose-response relationship engenders confidence and lends precision to the method.

Assays of pituitary gonadotropin at parturition and at ovulation, permit the following deduction. The pituitary gland shows a 4-fold decrease within 15 hours after parturition. Burdick and Whitney(1) had reported and we have confirmed (unpublished) that the shortest interval between administration of gonadotropin and ovulation is 12 hours. Therefore, it follows that the gonadotropin that elicits the first postpartum ovulation must be released within 3 hours after parturition.

Summary. The ovulatory responses of pregnant mice were evaluated with human chorionic gonadotropin. From these observations the end point response for this material was calculated to be 0.60 I.U. Assays of mouse pituitaries demonstrated a 4-fold decrease in gonadotropin content between parturition and the first postpartum ovulation. This decrease is interpreted as indicating that amounts of pituitary gonadotropin capable of inducing ovulation are released within 3 hours after parturition.

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A Simple Apparatus for Determining Basal Metabolism of Small Animals in Student Laboratory. (20719)

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Many types of apparatus have been described for determining basal metabolism of small animals(1-4), especially the rat. Haldane's open circuit method(5), in which oxygen consumption and carbon dioxide production are determined simultaneously, has been widely used with various modifications. Most of such equipment has been designed for research purposes and is too complex and expensive for routine use in student laboratory experiments when several sets of the apparatus are required.

The device described in this paper is economical, simple to construct and operate, and quite accurate for equipment of its simplicity. About 30 pieces of this apparatus have been in routine use in our laboratory during the last 3 years with good results.

The apparatus is shown schematically in Fig. 1. A standard wide-mouth, half-gallon Mason jar is secured to a wood base by 2 wire straps. The glass liner is removed from a zinc top[*] and a 13 mm hole drilled in the center. A 5 ml burette is made by etching lines on an 11 mm o.d. glass tube with 1 mm walls. This burette is sealed in the jar top by slipping a 14 mm o.d. rubber tube over the burette and pushing this gasket into the hole in the zinc top. The animal container is fabricated from 6 mm mesh hardware cloth in the shape of a cylinder closed at one end. The approximate dimensions shown in Fig. 1 are suitable for the adult rat but the size of the animal container can be varied to fit smaller or larger animals.

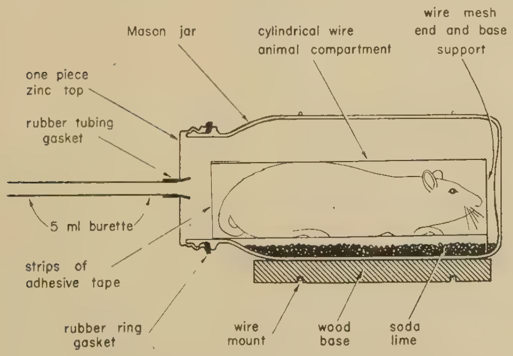

FIG. 1. Diagram of basal metabolism apparatus showing position of rat in animal container.

To determine the oxygen consumption of a rat the bottom of the chamber is covered with 8 mesh soda lime. The rat is placed in the animal container and the open end closed with strips of 50 mm adhesive tape. The cage is placed in the respiration chamber exercising care to prevent the animal from coming in contact with the soda lime. The chamber is flushed with oxygen and after wetting the walls of the burette with bubble soap,[†] the top is screwed on the chamber, using a standard rubber ring as a gasket to insure a tight seal. After waiting about ten minutes for temperature equilibration (the room temperature seldom varies more than 1°C during a laboratory period), a film of the soap is wiped across the end of the burette. As the rat uses oxygen and the carbon dioxide is absorbed by the soda lime, the film moves up the burette and the time taken for it to traverse the 5 ml volume is measured with a stop watch. Five determinations are usually made with the animal resting quietly in the cage and the mean is used for calculations.

Measurements of the rat's oxygen consumption are useful in connection with various exercises in a student laboratory. Typical results obtained in our laboratory are shown in Table I in which are listed the means and standard errors of the means of determinations of the oxygen consumption of adult rats (weighing 200-300 g) by 32 groups of students. The mean oxygen consumption of the control rats was 8.6 liter/m^2/hr which falls within 7% of the average value of collected data given by

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[*] If not available locally these can be obtained from Ball Brothers Co., Inc., Muncie, Indiana.

[†] The bubble soap is of the type used by children for blowing bubbles and is usually available locally. Films of this material are more durable than those made from ordinary soap.

TABLE I. Summary of Typical Results Obtained by a Class of Students Using the Simple Basal Metabolism Apparatus. Oxygen consumption values are means for 32 rats corrected to N.T.P.

Treatment	Oxygen consumption, liters/m^2/hr	Change from control	t	P
Control	8.6 ± .3			
Pentobarbital sodium (40 mg/kg)	5.6 ± .2	3.0	8.6	<.001
Tapazole (5 mg/kg/day)	7.8 ± .3	.8	2.0	.05

Griffith and Farris(6). After the oxygen consumption of each control animal was determined, the rat was injected intraperitoneally with 40 mg pentobarbital sodium[‡] per kg and further determinations made when the animal became comatose. As expected(7), the oxygen consumption of the rat under pentobarbital anesthesia was significantly lower which was determined statistically by using the test for the significance of the difference between means given by Goulden(8).

Another group of 32 rats had been given 5 mg Tapazole[§] per kg per day in their drinking water for 3 weeks. The oxygen consumption of these animals was determined by the same students in the same exercise. It is seen in Table I that administration of the antithyroid drug reduced the mean oxygen consumption by a small but statistically significant degree. Thus when used by an average group of students the simple basal metabolism apparatus herein described permits the measurement of even relatively small effects of drug administration.

[‡] The pentobarbital sodium was generously provided by Abbott Laboratories.

[§] The Tapazole (1-methyl-2-mercaptoimidazole) was generously provided by Eli Lilly and Co.

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Demonstration of β-Oxidation and Acetoacetate Formation in a Mouse Hepatoma.[*] (20720)

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The ability of normal tissues or their enzyme preparations to oxidize fatty acids via C_2 fragments (acetyl-S-Co$\bar{A}$) has been demonstrated convincingly by many workers. Until recently, however, fatty acid oxidation by neoplastic tissues received little attention. Weinhouse and coworkers, in a series of papers on the metabolism of neoplastic tissues(1-3), have provided evidence for the occurrence of β-oxidation of fatty acids and the existence of the tricarboxylic acid cycle in such tissues. An interesting aspect of their studies was the search for isotopic acetoacetate which might have accumulated during the oxidation of C^{14}-labeled fatty acids. The amounts of C^{14} recovered in the acetoacetate fraction apparently approached the lower limits of detection. In-

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[*] Supported by Cancer Research Funds of the University of California.

deed, as pointed out by Weinhouse *et al.*(3), their data did not exclude the possibility that there was *no ketogenesis at all* in tumor cells.

The formation and accumulation of acetoacetate by a tissue have obvious implications as to the manner by which that tissue oxidizes fatty acids. For this reason we sought to provide clear-cut evidence on acetoacetate formation in the case of tumors. It is shown here that during the oxidation of octanoate-1-C^{14} by slices of a hepatoma, isotopic acetoacetate not only is formed but also accumulates.

Methods. Labeled substrate. Sodium octanoate-1-C^{14} was obtained by carboxylation of the appropriate Grignard compound. *Nonisotopic acetoacetate.* The ethyl ester of acetoacetic acid was obtained commercially (Eastman Kodak), redistilled (b.p. 182-184°, uncorrected), and converted to sodium acetoacetate by hydrolysis with NaOH according to the procedure of Ljunggren(4). Ethyl alcohol, formed during hydrolysis, was not removed as it does not affect the use of the solution of sodium acetoacetate as carrier in the experiments described below. *In vitro procedures.* The tumor used in this study was an hepatocarcinoma, C_{954}, originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. It is maintained in this laboratory by serial transplantation in mice of the C_{57}-leaden strain. We are indebted to Mr. Carl Emanuel for the tumor tissue used in this study. Tumor slices, prepared freehand, were placed in cold isotonic saline solution, blotted gently on filter paper, weighed, and placed in incubation flasks containing 5.0 ml calcium-free phosphate buffer(5) and 5.0 μ moles of octanoate labeled with octanoate-1-C^{14}. Pertinent information concerning incubation conditions is given in Table I. At the end of the incubation period, 300 μM of the nonisotopic acetoacetate were added, and the reaction was stopped with H_2SO_4. *Analytical procedures.* The deproteinized ($Ca(OH)_2$ plus $CuSO_4$) buffer was subjected to the thermal decarboxylation procedure described elsewhere (6). Mercury-acetone precipitates(7) were filtered, while hot, at the purification stage(6) to minimize contamination by sparingly-soluble isotopic substances. Weighed samples of the mercury-acetone complex were combusted with Van Slyke-Folch reagent(8) (iodate-free), and the resulting CO_2 was collected in 0.5 N NaOH (CO_2-free). Saturated $BaCl_2$ solution was added to aliquots of carbonate solutions representing the acetone and carboxyl carbons of acetoacetate and the respiratory CO_2. The resulting $BaCO_3$ precipitates were mounted on filter paper and counted with a gas flow counter (helium saturated with ethyl alcohol at 0°) of about 20 per cent efficiency. Na_2CO_3 was added as carrier to solutions containing the respiratory CO_2 in order to obtain sufficient mass of $BaCO_3$ for counting. All counting data have been corrected for background and are corrected to a standard mass of 40.0 mg $BaCO_3$. The distribution of isotope in acetoacetate is represented by the C*O: C*OOH ratio. The ratio is expressed in two ways: (c.p.m. in acetone carbon)/(c.p.m. in carboxyl carbon) and 3(s.a. acetone carbon)/(s.a. carboxyl carbon), where s.a. (specific activity) represents c.p.m./mg $BaCO_3$.

Results and discussion. The results are shown in Table I. The percentages of the incubated C^{14} recovered in CO_2 plus acetoacetate varied from 13 to 17%. It is of interest to note that from 40 to 45% of the total isotope recovered in these two fractions resided in the acetoacetate. Negligible amounts of isotope were found in the various fractions from flasks containing boiled slices or no slices. This finding is of special importance since it clearly indicates that the mercury-acetone precipitates were obtained almost free of contamination by the labeled substrate.

The C*O:C*OOH ratio varied from 0.93 to 1.02 (average value, 0.95). Within experimental error, the ratio was the same whether calculated on a total c.p.m. or s.a. basis. Both should provide a valid measure of the C*O:C*OOH ratio since negligible amounts of isotope were found in iodoform (representing the methyl and methylene carbons of acetoacetate) prepared from an acetone sample.

Our finding that the isotope from octanoate-1-C^{14} appears in acetoacetate in amounts approaching those found in $C^{14}O_2$ is in contrast to that observed by Weinhouse *et al.*(3). The latter reported that litle or no isotopic acetoacetate accumulated in slices of a hepatoma or other neoplastic tissues incubated with octano-

TABLE I. Oxidation of Octanoate-1-C^{14} by Mouse Hepatoma, C_{954}. (500 mg slices; 5 ml phosphate buffer, pH 7.4; oxygen in gas phase; incubation time 90 minutes; t = 37.5°; KOH in center well; octanoate-1-C^{14} 0.001M, 2.9 × 10^5 c.p.m. incubated per flask.)

	C^{14}-c.p.m. recovered in:			C*O : C*OOH	
	CO_2	Acetoacetate†		Acetone	3 s.a. acetone carbon‡
Flask		Acetone	Carboxyl carbon	COOH	s.a. COOH
	A	B	C	B/C	
1	30200	9930 ± 40	10550 ± 150	.94	.93
2	22000	7385 ± 405	7960 ± 100	.93	.89
3	21200	8885 ± 585	8775 ± 125	1.01	1.02
Avg 1, 2, 3	24500	8730	9100	.96	.95
4 (boiled slices)	205	367	115		
5 (no slices)	96	214	117		

† Avg of 2 samples ± avg dev.

‡ s.a. = c.p.m. per mg $BaCO_3$.

ate-1-C^{14} as substrate. In order to demonstrate the formation of isotopic acetoacetate from butyrate-1-C^{14}, these workers added carrier acetoacetate at the start of the incubation so as to "trap" the ketone body. None of their studies, however, provided unequivocal evidence for the formation of ketone bodies. In experiments reported here, carrier acetoacetate was added at the *end* of the incubation period, and served merely to facilitate recovery and degradation of isotopic acetoacetate which accumulated.

In general, the metabolism of neoplastic tissues with respect to the end products of fatty acid oxidation resembles that of extrahepatic tissues(3). In our experiments, however, the tumor metabolized octanoate in a fashion reminiscent of the fasted liver(9). The tumor used here was taken from a mouse nearing the terminal state (3 weeks after implantation), and the degree of ketosis, perhaps reflecting the starved condition of the animal, may have served advantageously for the accumulation of large amounts of ketone bodies. Accumulation of sufficient amounts of isotopic acetoacetate for the determination of the isotope distribution in the ketone body enables us to draw the conclusion that fatty acid oxidation in the tumor proceeds by the generally accepted scheme of β-oxidation-cleavage-condensation. This conclusion is based on the following two findings: 1) accumulation of acetoacetate in which the isotope from octanoate-1-C^{14} appears in the expected carbons (carbonyl and carboxyl); and 2) observance of a C*O:C*OOH ratio which is not greater than unity(10).

Summary. Slices of a transplanted mouse hepatoma, C_{954}, oxidized octanoate-1-C^{14} to $C^{14}O_2$ and acetoacetate-1,3-C^{14}. Isotopic acetoacetate accumulated in quantities approaching that recovered in respiratory CO_2. The distribution of isotope in the ketone body is consistent with the view that the short-chain fatty acid was degraded by β-oxidation, the resulting C_2 fragments condensing to form acetoacetate as well as being oxidized to CO_2.

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Natural Occurrence of *Leptospira ballum* in Rural House Mice and in an Opossum. (20721)

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Borg-Peterson(1) first isolated the serotype strain (mus 127) of *Leptospira ballum* from the urine of a rural house mouse, *Mus musculus specilegus,* trapped in Southern Jutland, Denmark, and established its distinct leptospiral serologic relationship. Subsequently, Wolff, *et al.*(2) traced the source of an accidental human laboratory infection with *L. ballum* to the natural occurrence of these organisms in the urine of the laboratory white mice. The inapparent infectivity of these leptospires for white mice and the persistence of the urinary carrier stage during the entire life span of the host have been noted(1,2).

In the Western hemisphere, leptospires of the serotype *ballum* have been recovered from naturally occurring infections in human beings and Norway rats in Puerto Rico(3,4), Norway rats trapped in British Columbia(4), stock laboratory white mice at the Army Medical Service Graduate School, Washington, D. C. (5) and the Rocky Mountain Laboratories of the U. S. Public Health Service(6).

In this paper, the authors report naturally occurring infection with *L. ballum* in domestic mice (*Mus musculus*) and in an opossum (*Didelphis virginiana*), trapped in a rural area near Middleburg, Virginia, coincident to an epidemiological investigation of possible wild life carriers of *L. pomona* in an endemic area of bovine leptospirosis.

Methods. Animals were trapped by the Virginia Cooperative Wildlife Research Unit, Virginia Polytechnic Institute Biology Department, Blacksburg, in cooperation with Dr. Wilson C. Bell, in and around pastureland and housing facilities frequented by domestic ruminants. Trapped animals were delivered to the Veterinary Division, Army Medical Service Graduate School, where they were bled for serum specimens. The animals sacrificed, were then autopsied and appropriate tissues cultured for leptospires. Fletcher's and Schuffner's leptospiral media(7) were inoculated with 1 drop (ca .03 ml) of a 10% kidney suspension in physiological saline solution. Urine or saline washings were aspirated aseptically from the bladder, examined by dark field microscopy and cultured for leptospires. Cultures were incubated at 30°C for 30 days. *Identification* of the isolated strains was based upon the cross agglutination-lysis pattern of these organisms employed as antigens against 18 type-leptospiral immune rabbit serums, selected on the basis of previous serologic classification studies. Similarly the serums from all host animals except house mice were tested for the presence of leptospiral antibodies against a screening battery of type-leptospiral antigens(4). The technic of the agglutination-lysis test employed is described elsewhere(7).

Results. The following species and numbers of wild animals were trapped and studied: 1 skunk (*Mephitis mephitis*); 3 squirrels (*Sciurus carolinensis*); 2 wild rabbits (*Sylvilagus floridanus*); 1 muskrat (*Ondatra zibethica*); 1 deer mouse (*Peromyscus sp.*); 2 opossums (*Didelphis virginiana*); 27 house mice (*Mus musculus*).

Post mortem examination of tissue revealed no gross pathology, except for occasional tapeworm cysts in the livers from several house mice. Examination of sampled serums from all animals but the house mice did not disclose leptospiral antibodies, except for 1 opossum which showed an agglutination-lysis titer of 1:400 against *L. ballum.*

Dark-field microscopic examinations of urine or bladder saline washings of 10 house mice disclosed the presence of leptospires. Positive leptospiral cultures of kidney suspensions or urine were obtained from 9 of these 10 house mice, and from 6 additional house mice. A leptospiral isolate was also obtained from the opossum which had an agglutinin titer against *L. ballum.* The cross agglutination pattern of all the isolates were identical within the limits of the methods employed. The results of the

TABLE I. Cross Agglutination-Lysis Reactions of Isolates.

Antiserum	Homologous titer	Antigens	
		L. ballum[f]	Isolates
L. andaman[a]	102400	—	—
semarang[a]	409600	—	—
djasiman[a]	25600	—	—
ballico[a]	25600	—	—
australis B[a]	25600	—	100
pomona[b]	25600	—	—
canicola[b]	6400	400	400
djatzi[b,h]	102400	—	—
alexi[b,i]	6400	100	400
hyos[c]	102400	—	—
sentot[d]	409600	—	—
medanensis HC[d]	102400	—	—
autumnalis[e]	25600	—	—
ballum[f]	25600	25600	25600
grippotyphosa[f]	102400	—	—
icterohemorrhagiae AB[g]	25600	—	—

[a] Received from G. W. Hooper Foundation, San Francisco, Calif. [b] Isolated by Veterinary Division, AMSGS, Walter Reed Army Medical Center, Washington, D. C. [c] Received from the Inst. Bacteriologica Malbran, Buenos Aires. [d] Received from the Indisch. Inst., Amsterdam. [e] Received from Nat. Inst. of Health, Bethesda, Md. [f] Received from Pasteur Inst., Paris. [g] Received from the Inst. Trop. Med., Leiden. [h] Member of the serogroup *bataviae*. [i] Member of the serogroup *pyrogenes*.

serologic typing of the isolates are shown in Table I. Titers are expressed as the reciprocals of the highest dilution showing a positive reaction. The strains show the typical cross-agglutination reactions peculiar to *L. ballum*.

Summary. In a search for wildlife carriers of *L. pomona*, various species of mammals were trapped in an endemic area of bovine leptospirosis. Direct dark-ground microscopic examinations of urine or bladder saline washings disclosed the presence of leptospires in 10 of the 37 house mice examined. Leptospiral cultural isolates were obtained from 9 of these 10 house mice. The recovery of leptospires from 6 additional house mice and from 1 of 2 opossums emphasizes the importance of cultural methods in any survey for leptospirosis. All isolates, serologically typed on the basis of a cross agglutination-lysis pattern, employing 18 type leptospiral immune rabbit serums, belonged to the serotype *L. ballum*. The failure to detect *L. pomona* in the rodent and other wild life population in an endemic area of bovine leptospirosis affords ancillary evidence that the natural hosts of *L. pomona* leptospirosis are to be found primarily in the livestock population. To the authors' knowledge, this is the first report of leptospirosis in the opossum.

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Pantothenic Acid Derivatives and Growth of *Acetobacter suboxydans*.*

(20722)

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Acetobacter suboxydans was the first microorganism(1) to be found responsive to bound forms of pantothenic acid (PA).† The conjugates PAC†(2) and coenzyme A† (Co A) (3,4) were both more active for this organism than is free PA. However, when Co A was filter sterilized, no activity could be observed. This paper will report the behavior of panteth(e)ine† (*Lactobacillus bulgaricus* factor; LBF), LBF-γ-phosphate† and α,γ-diphosphate,† pantothenyl cystine† (PA-cystine) and Co A after heat or filter sterilization, in the growth of this organism.

Experimental. The medium and assay method were the same as previously reported (5), except for the sterilization. Heating of all samples was done by autoclaving at 120° for 10 minutes, whereas filter sterilization was accomplished by use of Seitz or "ultra-fine" sintered glass filters. *A. suboxydans* ATCC strain 621 was used. Co A, approximately 80% pure, was purchased from Pabst Laboratories, Inc. LBF samples, prepared by three different methods, were obtained from 3 laboratories, as follows: sample (a), prepared by condensation of pantothenic azide and aminoethyl mercaptan(6), from Parke, Davis and Co.; sample (b), prepared by the method of Baddiley and Thain(7), from these authors; and sample (c), prepared by the method developed in this laboratory(8). LBF-γ-phosphate was prepared by the method of Baddiley and Thain(9) and furnished by them. Pantothenyl cystine was supplied as the disodium salt by J. F. Cavalla, Parke, Davis and Co., London. Crude LBF-α,γ-diphosphate† was prepared by condensing methyl bis (diphenylphospho)-pantothenate(10) with aminoethyl mercaptan. After reduction, a crude sample of 30-40% purity was obtained by fractionation with $Ba(OH)_2$.

Results. LBF was first tested for its growth-promoting effect in the absence of PA by heat sterilizing the sample with the medium in the usual manner. The results are summarized in Fig. 1-A. It is evident that LBF is more active than free PA. The effect could not be duplicated by supplementing the free vitamin with aminoethyl mercaptan. This fact indicated that the biosynthesis of the amide linkage between PA and aminoethyl mercaptan might be a limiting reaction in the growth of the microorganism. LBF prepared by different methods in different laboratories showed practically the same activity. *LBF-γ-phosphate* has been shown as an intermediate(11) in Co A formation in liver. Accordingly, its stimulatory behavior was tested. When the sample was sterilized along with the medium, as shown in Fig. 1-A, the phosphate was much superior to PA and even slightly more active than Co. A.

However, when LBF-γ-phosphate was filter sterilized or its aqueous solution was heated and then added aseptically to the (heated) growth medium, no activity could be demonstrated (Fig. 1-B). Likewise, *Co A* was inactive after filter sterilization (Fig. 1-C). The activity of LBF was also less after filtering than after heating, whereas PA gave the same response after either treatment (Fig. 1-A).

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† The following abbreviations will be used throughout this paper:

pantothenic acid = PA
" " conjugate = PAC
coenzyme A = Co A
panteth(e)ine = *Lactobacillus bulgaricus* factor = LBF
panteth(e)ine-γ-phosphate = LBF-γ-phosphate
" α, γ-diphosphate = LBF diphosphate
pantothenyl cyst(e)ine = PA-cyst(e)ine

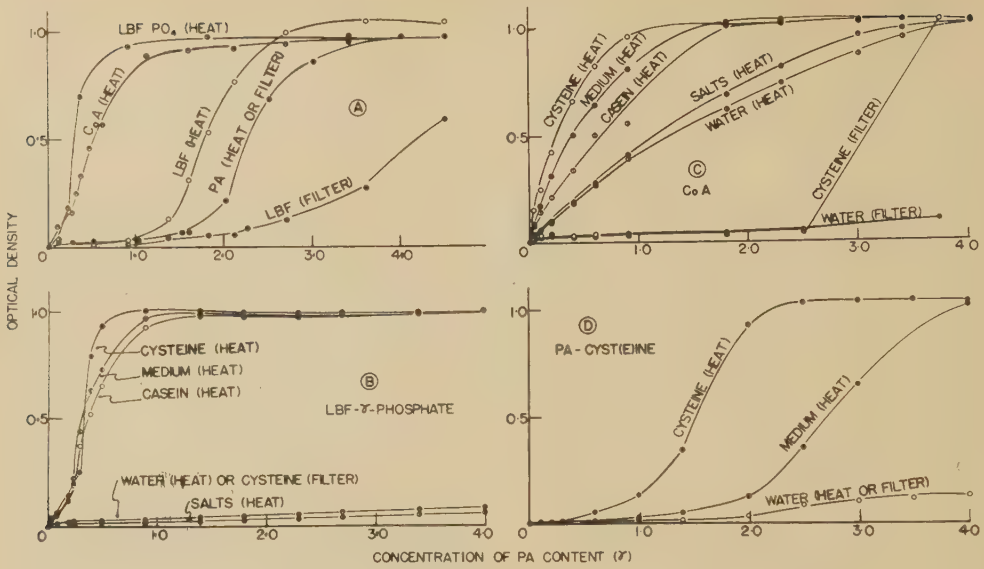

FIG. 1. Growth promoting effects of various bound forms of pantothenic acid (PA†) for *A. suboxydans*. The concentration of each PA derivative is given in terms of its PA content. 1-A: "Heat" = compound autoclaved with basal medium; "filter" = compound sterilized by filtration, added aseptically to heat-sterilized medium. 1-B, 1-C and 1-D: Growth factor mixed with each listed component (concentration equal to that present in medium), heated or filtered as indicated, then combined with remaining ingredients of medium; the latter were heat sterilized. L-Cysteine was added at 2 mg/10 ml without adjustment of amino acids in medium.

The component(s) in the medium responsible for the activation of these compounds were then sought. The results are shown in Fig. 1-B and 1-C. Co A could be partially activated by heating in water and almost fully by heating with casein hydrolysate. High activity could also be produced for LBF-γ-phosphate by heating with casein hydrolysate. When various amino acids were tested for activating ability, it was found that cysteine could replace casein hydrolysate. However, when the samples were filter sterilized with cysteine, the growth stimulation was much lower. Ascorbic acid and thioglycolic acid were much less effective than cysteine, either in a heated or filtered mixture. Hydrogen sulfide-treated Co A or LBF-γ-phosphate were stimulatory, but became less so as complete removal of hydrogen sulfide was achieved. The reactivation of Co A or LBF-γ-phosphate by heating with cysteine (medium autoclaved separately) suggests that only the reduced forms are utilizable. The reduction is obviously more favorable at an elevated temperature. The somewhat greater ease of reactivation of Co A over LBF-γ-phosphate in various heat treatments indicates a difference in their redox potentials, and of activation energies in the reduction. This non-utilizability of the oxidized forms is in line with the fact that a reduced compound, either cysteine or glutathione, is required in all enzymic experiments with Co A.

Recently Brown and Snell(12) reported that PA-cysteine was more active than the free vitamin for *A. suboxydans,* and that the reduced form was also more active than the oxidized. They concluded that PA-cysteine was a precursor of LBF and Co A. It was therefore of interest to compare whether PA-cyst(e)ine showed the same trend of behavior as LBF-γ-phosphate and Co A under various conditions of sterilization. The results, which are summarized in Fig. 1-D, indicate a pattern similar to those of Co A and LBF-γ-phosphate, although with PA-cystine the basal medium was much less effective than cysteine as an activating agent. This may have been due either to the difference of redox potentials of PA-cystine, LBF-γ-phosphate and Co A, or to insufficient reducing agent in the medium. PA-cysteine was less active for growth than

LBF-γ-phosphate in these experiments.

Other derivatives. Pantothenic acid phosphates(13,14) are inactive for *A. suboxydans*, possibly due to cell impermeability. The phosphates of LBF, on the other hand, appear to be freely permeable in view of the foregoing experiments with LBF-γ-phosphate and with *LBF-α,γ-diphosphate*. The latter compound was found in a heated medium (data not shown) to possess 30 to 50% of the equivalent activity of pantothenic acid, at concentrations of the diphosphate ranging from 20 to 120 γ of the preparation per 10 ml culture.

The order of effectiveness of PA derivatives for *A. suboxydans* growth is thus, roughly, Co A = LBF-γ-phosphate >LBF = PA-cysteine (approx.) >PA = pantoic acid(15) >LBF-diphosphate >PA-α- or γ-phosphate (→0), with Co A and LBF-γ-phosphate about 10 to 20 times as active as PA. The parallelism between activity and increasing structural complexity of the PA derivative, up to the level of LBF-γ-phosphate, points to a restricted synthetic ability of the organism for 1) the amide linkage between aminoethyl mercaptan with the carboxyl group of PA, and for 2) the phosphorylation of the γ-hydroxy group. If the assumption is valid that Co A is the only physiologically active form of the vitamin, the syntheses of the other moieties of Co A from LBF-γ-phosphate are apparently accomplished easily in this organism.

Summary. 1. Various derivatives of pantothenic acid have been compared for growth promoting activity in *A. suboxydans*. The order of effectiveness is roughly Co A† = LBF-γ-phosphate >LBF = PA-cysteine (approx.) >PA = pantoic acid >LBF-diphosphate >PA phosphates, with Co A and LBF-γ-phosphate about 10 to 20 times as active as PA. The derivatives containing –SH groups appear to be active principally, or even exclusively, in the reduced state. 2. It is concluded that the "*A. suboxydans* stimulatory factor" is multiple in nature, but is not identical with pantothenic acid phosphates.

The authors wish to thank Mrs. Marion McDonald and Miss Gwendolyn Cahill for valuable technical assistance, and the various persons listed above for samples of compounds tested.

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Isotopic Studies of the Body Potassium Content in Thyrotoxicosis.* (20723)

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Hyperthyroidism is characterized by an increase in the rate of all bodily functions. Among the more striking clinical features of the disease are generalized muscular weakness and loss of body weight. Degenerative changes in the skeletal muscle have been demonstrated by histologic technics. Creatine and nitrogen metabolism are altered. All of these observations indicate that there is some abnormality of muscle metabolism in this disease. Since muscle tissue contains the largest body store of potassium, a disturbance in potassium metabolism might also be suspected in the hyperthyroid state.

Because of the technical difficulties involved in the measurement of soluble cations, very few data concerning the metabolism of potassium in hyperthyroidism are available. The introduction of flame spectrophotometry and the availability of synthetic radioactive isotopes have partially overcome these technical limitations. Direct measurement of the body store of potassium in intact animals is now possible by the use of a radioactive isotope of the atom-K^{42}(1). The ranges of values for the "total exchangeable potassium content (Ke)" in normal young men(1) and women(2,3), and also in various disease states(4), have been previously reported. The purpose of the present study was to determine the exchangeable potassium content in subjects with untreated hyperthyroidism.

Material. Subjects. Thirteen hospitalized subjects—6 males and 7 females—with the diagnosis of hyperthyroidism were studied; their ages ranged from 21 to 68 years. All subjects showed unequivocal clinical symptoms and signs of increased thyroid activity at the time of study. The clinical impression was confirmed in 12 instances by the finding of a basal metabolic rate of +25% or greater, or by a protein bound iodine determination showing a level higher than 10 μg per 100 cc of serum (Table I). In the 13th subject (Case 2), the serum cholesterol concentration was 188 mg per 100 cc. *Isotopes. Isotopic* potassium (K^{42}) was prepared for injection in the manner described by Corsa and his coworkers(1). The subjects were given 100 μc of K^{42} contained in 1.5 or 3 milliequivalents of potassium chloride solution, depending on the age of the solution.

Exchangeable potassium content (*Ke*). The determination of the exchangeable potassium content (Ke) was performed as soon as possible after the subject's admission to the hospital and prior to the initiation of therapy. Each subject received an intravenous injection of radioactive potassium by calibrated syringe between 7:30 and 9 a.m. The patient was then asked to void and to discard this specimen; all urine voided thereafter until 7 a.m. the following day was collected; and the K^{42} content of the pooled specimen was determined. Determinations of the specific activity were made on 2 spot samples of urine obtained at 8 and 9 a.m. on the day after injection. The following formula was used to calculate the value for the exchangeable potassium content of the body:

$$Ke = \frac{Ki^{42} - Ko^{42}}{Ku^{42}/Ku^{39}}.$$

Ke = quantity of exchangeable potassium in milliequivalents.

Ki^{42} = quantity of radiopotassium administered (arbitrary units).

Ko^{42} = quantity of radiopotassium excreted in pooled specimen of urine.

Ku^{42} = conc. of radiopotassium in spot samples.

Ku^{39} = conc. of nonisotopic potassium in spot samples.

Ku^{42}/Ku^{39} = mean specific activity of the 2 spot samples.

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K^{42} was supplied by the National Laboratories, Oak Ridge, Tenn., on allocation from the U. S. Atomic Energy Commission.

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TABLE I. The Exchangeable Potassium Content in Thyrotoxicosis, 13 Subjects.

Sex	Age (yr)	Ke (meq)	Wt (kg)	Ke/wt (meq/kg)	BMR (%)	PBI (μg %)	Wt loss (kg in mo)	
♂	48	1421	75.5	18.8		26.9	59.1	12
♂	68	1413	45.5	31.1			22.7	9
♂	39	1491	56.4	26.4	+76		13.6	12
♂	27	1534	70.5	21.8	+98		13.6	3
♂	68	1660	60.0	27.7	+43		Not known	
♂	21	2344	61.1	38.4	+25		None	12
♀	54	981	45.8	21.4	+36		13.6	24
♀	58	1556	59.1	26.3		16.8	Not known	
♀	21	1895	55.5	34.1	+77		7.7	6
♀	54	1513	42.0	36.0	+40	11.2	13.6	8
♀	23	1369	46.4	29.5	+100		4.0	1
♀	50	1909	62.3	30.6		10.7	6.8	1
♀	59	1471	41.8	35.2	+41		65.0	24

Preliminary studies confirmed the observation of Corsa and his associates(1) that the specific activity of K^{42} in the urine reaches an equilibrium, in diseased as well as in normal subjects, within 24 hours(4).

Measurement of radioactivity. The activity of the urine and serum specimens was determined with a dipping tube and a scaling circuit. A total of 10,240 counts was made on each sample. All counting rates were at least 10 times background, and were usually in the range of 500 to 3000 per minute. At these counting rates, no correction for coincidence loss was necessary. All determinations were corrected for decay. The total *potassium* concentration in the urine was determined by flame spectrophotometry, using the method of Mosher and his coworkers(5).

Results. The reported range for Ke in normal males is 2770 to 4470 meq. with a mean of 3402 meq., or 35.6 to 53.6 meq/kg with a mean of 46.3 meq/kg(1). For normal females the range in one series(2) was 1374 to 2164 meq. with a mean of 1776 meq., or 25.1 to 37.9 meq/kg with a mean of 31.5 meq/kg; in another series(3), it was 28.0 to 47.2 meq/kg with a mean of 40.7 meq/kg.

Males. In the present study, all of the absolute Ke values in the males were below the normal range (Table I). When the Ke values were related to body weight, all but one were subnormal. The one normal value was found in a subject (Case 6) who had no history of weight loss. The individual (Case 1) who had lost the greatest amount of weight had the lowest value for Ke/wt. In general, therefore, there was a fair correlation between the Ke and the decrease in body weight.

Females. In the females the relation between weight loss, clinical symptoms and the Ke values was not as obvious as in the males. Of the 7 subjects, only one (Case 7) had subnormal values for Ke and Ke/wt (Table I). Although 5 of the remaining 6 gave histories of weight loss, the Ke values were within the normal range. One of these subjects (Case 13) had lost more weight than any other patient studied.

Comment. The results which were obtained in the male patients in the present study are compatible with the following interpretation: While abnormal production of the thyroid hormone increases the rate of catabolism of all body tissues, muscle tissue is most rapidly broken down. Consequently the decrease in body weight which occurs in this disease is due largely to a reduction in the body's muscle mass. As the muscle mass is reduced, the total exchangeable potassium content is decreased. Since this decrease in Ke is due primarily to a reduction in muscle mass, it does not necessarily indicate an intracellular metabolic deficiency (a decrease in the intracellular concentration of potassium). (In contrast, the metabolic abnormality which has been previously found, for example, in poorly controlled diabetes(6), apparently produces a true intracellular deficiency).

The variability of the results obtained in the females is more difficult to interpret, but can probably be explained on the basis of the differences in body composition normally found between males and females. In health, the bodies of women contain proportionately more fat and less muscle tissue than those of men(7); the lower values for Ke/wt in normal females are attributed to this factor. When thyrotoxicosis occurs in women, fat is catabolized in preference to muscle tissue, so that the relative fat content decreases; hence, the Ke/wt value initially would tend to remain unchanged or actually to increase. With more severe or protracted thyrotoxicosis, which exhausts the store of fat, a proportionately greater quantity of muscle tissue is eventually catabolized and the Ke/wt value decreases to subnormal levels. Such may have been the case in subject 7.

The results of the present study suggest that the alterations in the exchangeable potassium content noted in thyrotoxicosis can be adequately explained on the basis of the nonspecific catabolic effects of increased thyroid activity. There is no evidence in the present study to suggest a defect in the intracellular metabolism of potassium.

Summary. 1. A radioactive isotope of potassium (K^{42}) was used to determine the exchangeable potassium content (Ke) in 13 subjects with untreated thyrotoxicosis. The Ke values were lower in untreated thyrotoxic males than in normal male subjects. It is concluded that the low Ke values could be attributed to a decrease in the total muscle mass of the body, resulting from increased catabolism of body tissue. 2. In contrast to the results found in men, the values for Ke/wt in untreated women were normal, with one exception. This sex difference has been attributed to the dissimilarity in body composition normally found between males and females. It is postulated that in women mild or early thyrotoxicosis decreases the relative fat content of the body. Muscle tissue is extensively catabolized only after the store of fat has been depleted.

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Aging Changes in Nucleic Acid and Protein-Forming Systems of the Virgin Mouse Uterus.* (20724)

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Several investigators(1-3) have established that nucleic acid concentration is much higher in rapidly growing embryonic tissue than in the corresponding adult tissue. Schulz(4) attempted to follow such a change in the mouse liver with aging but was unable to detect any changes in the concentration of either ribonucleic acid (RNA) or desoxyribonucleic acid (DNA). He expressed his results in terms of concentration of the nucleic acids per mg tissue. Since the extreme chromosomal polyploidy of liver tissue makes the use of RNA/DNA ratios of little use in so far as using them as an estimate of relative RNA per cell, it is possible that any change, if it did occur at the

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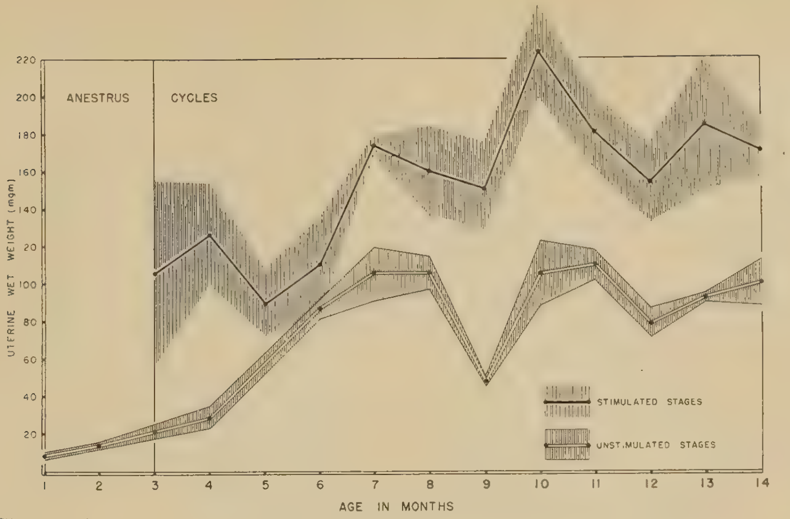

FIG. 1. Changes in wet weights of uteri from 1 to 14 mo of age. Stand. errors of the means represented by shaded areas.

cellular level, may have been masked. Earlier work(5-7) has shown that the use of the ratio between ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) gives useful information on the growth activity of the uterus in certain experimental and normal physiologic conditions. It was considered of interest to make a systematic study of these changes during the early maturation and subsequent aging of the mouse uterus to determine whether or not the aging process itself might be accompanied by a decrease in the amount of RNA per cell. In the course of this experiment data have been collected which indicate that there is a progressive decrease in the amount of RNA per cell in the uterus with age, and also that this cellular RNA, as expressed by the RNA/DNA ratio, bears a definite relationship to the uterine water content and to the amount of protein synthesized.

Methods. To eliminate any possible effects of pregnancy on the maturation process only virgin females of strain 129 mice were used in the following study. Groups of mice were sacrificed at monthly intervals from 1 to 14 months of age, a total of 247 animals being used. Animals were maintained on Purina Fox Checkers and water *ad libitum* and were routinely sacrificed between 8:30 and 10:30 A.M. to minimize any possible effects due to the time of feeding. At the time of sacrifice the stage of the estrous cycle was determined by a vaginal smear and the animals classified as being in proestrus, estrus, early metestrus, late metestrus or diestrus. Since not enough animals at any one stage of the estrous cycle were obtained at each age, the animals were grouped into two major classes, stimulated stages (proestrus, estrus, and early metestrus) and unstimulated stages (late metestrus and diestrus). For these reasons each of these two major groups was rather heterogeneous and so no attempt at a statistical analysis of differences between the stimulated and unstimulated stages at each age was made. The uteri were removed immediately after sacrifice and dissected free of adherring connective tissue. After weighing on a Roller-Smith torsion balance, they were homogenized in cold 5% trichloroacetic acid and processed prior to analysis as has been described previously(7). Individual uterine samples were then weighed on an analytical balance. Water content was calculated from the initial wet weight and the weight of these dehydrated and delipidated uteri. Prior to 3 months the uteri were too small for complete analysis on individual uteri and so uteri were pooled. Sub-samples of the dried residue were removed for protein nitrogen analysis by a micro-Kjeldahl method(8) and nucleic acids extracted from the remainder by the Schneider procedure(9). DNA in the extract was determined by the Dische(10) reaction and RNA by the Mejbaum(11) method.

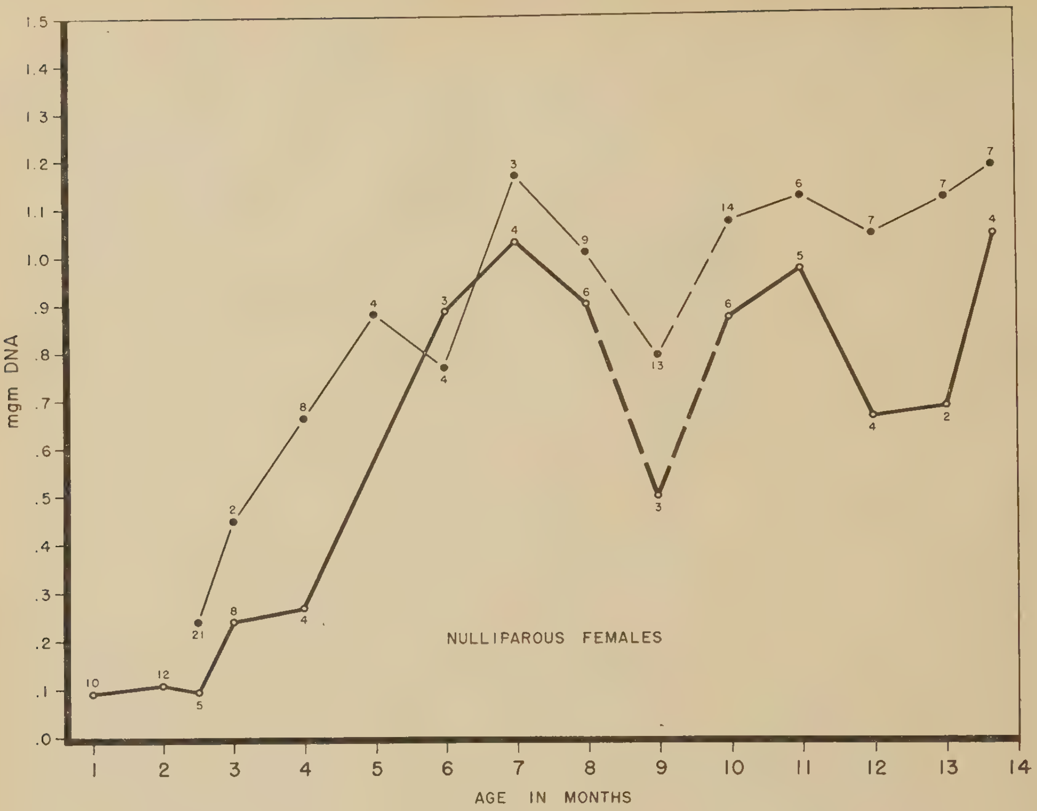

FIG. 2. Increase in amount of total uterine DNA from 1 to 14 mo of age in virgin mice. Heavy line—DNA at unstimulated stages; light line—DNA at stimulated stages. Numbers above each point indicate number of analyses at each point. Drop in the curve evident at 9 mo is at present unexplainable and is probably of no significance.

Results. Changes in mean uterine weight are presented in Fig. 1. However, it was felt that growth of the uteri could be followed more accurately by means of total uterine DNA as an indication of relative numbers of cells. Relative amounts of RNA per average cell were estimated by the use of the RNA/DNA ratio as proposed by Davidson(12). This assumes that the nuclear DNA remains constant within the cells and can be used validly in tissue where there is no large amount of polyploidy. That the DNA content of the cells of the rodent uterus remains constant during various conditions of hormonal stimulation has been shown by Alfert and Bern(13) using microspectrophotometric technics.

The increment of total uterine DNA is shown in Fig. 2. The lighter line represents the amount of DNA at the stimulated stages and the heavy line the amount at the unstimulated stages. At all ages the DNA values at the stimulated stages are somewhat greater than at the unstimulated stages. This is probably due to some extent to the infiltration of leukocytes during the late estrus and metestrus stages as well as to a slight increase in the number of uterine cells at these stages. From one to 2½ months the animals were in an anestrous condition so that only the values for unstimulated uteri are shown in this early period. It will be noted that the period of most rapid growth is from 2½ to 6 months.

Changes in the RNA/DNA ratio of these uteri during this same period of time are shown in Fig. 3. In all cases the ratio is higher at the stimulated stages than at the unstimulated stages. It is interesting that the greatest values of this ratio occur at the stimulated stages during this period of early maturation (*i.e.* from 2½ to 4 months). This coincides with the early period of most rapid uterine growth as evidenced by the increase in total DNA (Fig.

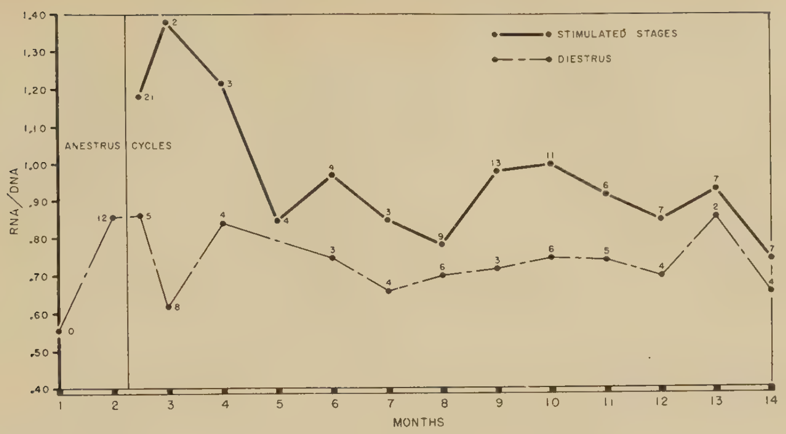

FIG. 3. Changes in RNA/DNA ratio of uterus at stimulated stages (solid line) and unstimu lated, ''diestrus'' stages (broken line) of estrous cycle from 1 to 14 mo of age. Numbers above each point indicate number of analyses at each point.

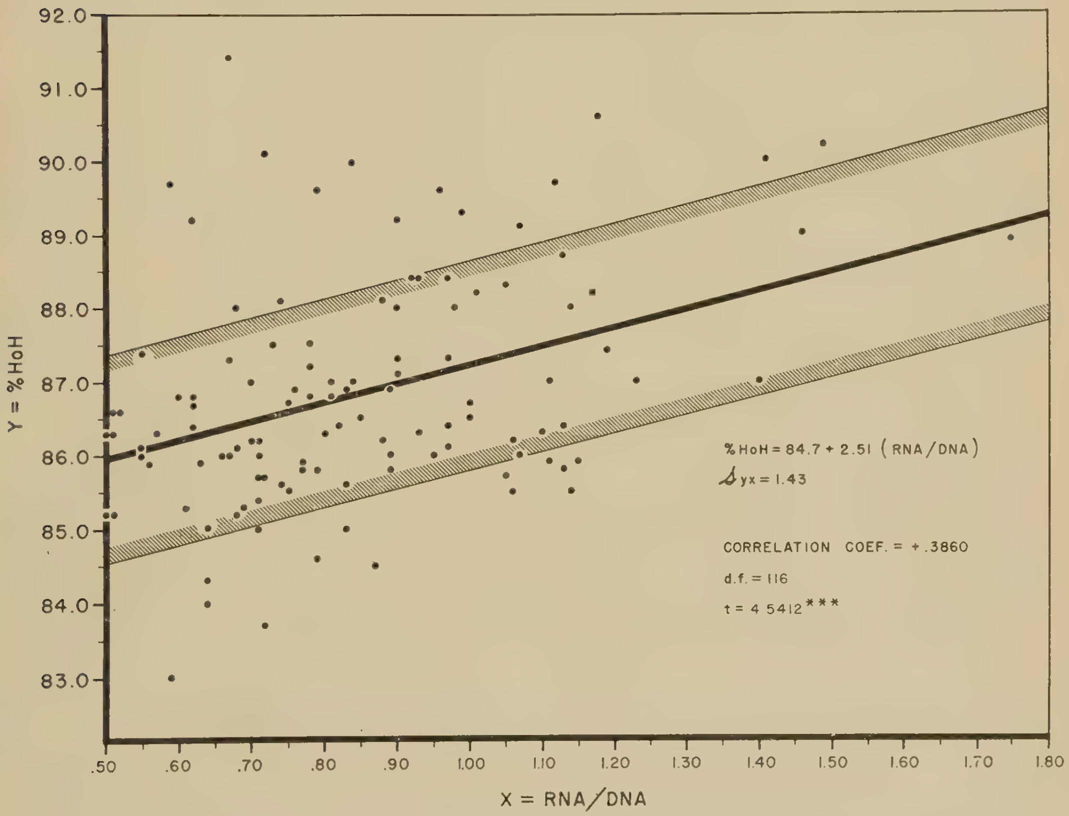

FIG. 4. Regression line of uterine water content on RNA/DNA ratio of uteri of mice from 3 to 14 mo of age. Stand. error of regression line is indicated by shaded outlines.

2). These animals showed vaginal cycles after 2½ months of age. However, corpora lutea were not found in the ovaries and ova not found in the oviducts upon histologic examination until this period of early rapid growth of the uterus was completed at 5 months of age(14). After this early period of rapid growth, the elevation of the RNA/

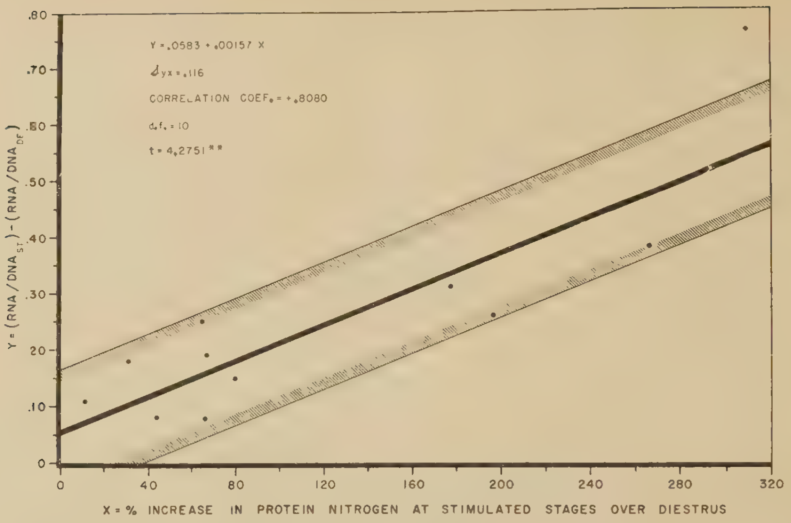

FIG. 5. Regression line of amount of increase in RNA/DNA ratio of uterus on percent increase in protein nitrogen at stimulated stages over the unstimulated "diestrus" stages. Stand. error of regression line is indicated by shaded outlines.

DNA ratio at the stimulated stages is not so pronounced and remains fairly constant until near the end of the age span studied here when it again appears to fall.

These changes in uterine RNA/DNA are paralleled by the changes in the uterine water content and in the amount of protein synthesized during the stimulated stages of the estrous cycle at any age. This results in the appearance of 1) a high degree of correlation between uterine water content and the uterine RNA/DNA ratio (Fig. 4), and 2) a correlation between *absolute increase* in the RNA/DNA ratio at the stimulated stages over the diestrus or unstimulated stages at a particular age and the amount of protein nitrogen synthesized (the percent increase of total protein nitrogen at the stimulated stages over that present at the unstimulated stages) (Fig. 5). For the correlation of RNA/DNA with uterine water content, values obtained on individual uterine samples of animals from 3 to 14 months were used. It was not possible to do this when the amount of increase in the RNA/DNA ratio at stimulated stages over unstimulated stages was to be correlated with the percentage increase in protein nitrogen. In this latter case, the average RNA/DNA ratio at the unstimulated stages was subtracted from the average ratio at the stimulated stages at each age to give the absolute amount of increase of the ratio. This value was then paired with the percentage increase of protein nitrogen at stimulated stages over unstimulated stages at each age, again utilizing average values. Such an analysis shows that a positive relationship exists between the RNA/DNA value of a single uterus and its water content ($r = +.3860$) and that the "t"-value of this correlation coefficient has a probability of less than .001. Likewise, the absolute increase in the RNA/DNA ratio is related to the percent of protein nitrogen synthesized ($r = +.8080$) and that the "t"-value of this correlation coefficient has a probability of less than .01.

Discussion. Since the early work of Caspersson(15) and Brachet(16) much evidence has been accumulated to show that ribonucleic acid in particular is linked in some manner with the synthesis of protein. The synthesis of new protein is undoubtedly one of the true requisites of growth and is therefore of importance in the maturation of aging of any organ. The uterus is a particularly interesting organ in which to study these changes since it is subjected to cyclic growth stimuli by the ovarian hormones. The data presented above seem to indicate that the early maturation and growth of the uterus of the mouse (from 2½ to 5 months in strain 129) occurs cyclically under the repeated stimuli of the early estrous cycles with their accompanying high levels of estrogen. This is to be assumed from the fact that during this early period the very high levels reached by the RNA/DNA ratio of the uterus characteristic of these early months are reached only during the stimulated stages of the estrous cycle. The RNA/DNA values for the unstimulated stages of the cycle remain constant at about .70 to .80 throughout the entire period studied here (from 2½ to 14 months). The correlation obtained here between the increase in the amount of RNA per cell (the RNA/DNA ratio) and the percentage increase in protein nitrogen during the stimulated stages is merely further confirmation of the increase of cellular RNA at times of protein synthesis which has been noted previously by many other investigators in other types of biologic materials. That there might be a correlation between cellular RNA and water content might be expected from our general recognition of the fact that increase in water content of a tissue generally occurs during active growth(17,18). However, such a correlation between the RNA/DNA ratio and water content has not been demonstrated specifically previously.

Summary. Changes in the total DNA, protein nitrogen, the RNA/DNA ratio and water content have been studied in the uteri of mice of strain 129 from 1 to 14 months of age. RNA/DNA (an estimate of relative RNA per cell) at the stimulated stages of the estrous cycle is maximal at the time of maximal uterine growth, between 2½ and 5 months of age. The RNA/DNA of the uterus at the stimulated stages is higher than at diestrus at all ages. However, the differences between the diestrous values and that reached by the ratio at the stimulated stages slowly decreases with age. The cellular RNA content is significantly correlated with the water content of the uterus under the conditions studied here and is also positively correlated with the amount of protein nitrogen synthesized.

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Inactivation of Histamine by Lung Tissue from Histamine-Sensitive and Histamine-Resistant Mice.* (20725)

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Mice are naturally resistant to histamine. Inoculation with *H. pertussis* vaccine greatly increases the sensitivity of these animals to histamine(1,2), to bacterial vaccines(2), and to anaphylactic shock(2,3). It appeared possible that *H. pertussis* vaccine might sensitize mice to the lethal effects of histamine by altering the ability of the animals to inactivate

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this agent. The tissues of most animals contain an enzyme histaminase which will detoxify histamine. Mouse lung is a rich source of this enzyme(4).

Materials and methods. Adult male white mice weighing 16 to 23 g were employed. A group of mice was injected with 0.2, 0.1, and 0.1 ml of *H. pertussis* vaccine[†] on each of 3 successive days. Six days after the last injection, 4 of the pertussis-inoculated mice and 4 uninoculated animals were challenged intraperitoneally with 50 mg/kg of histamine dihydrochloride. If all pertussis-inoculated mice died and all control mice survived, the remaining pertussis-injected animals were considered to be histamine-sensitive. The LD50 of histamine dihydrochloride in pertussis-inoculated mice was found to be 4.7 mg/kg; in uninoculated mice, 570 mg/kg. The histamine-inactivating power of mouse lung was determined by the method of Rose, Karady, and Browne(5), with certain modifications. The lungs of 5 histamine-sensitive or 5 histamine-resistant mice were pooled, weighed and ground. The ground tissue was placed in an Erlenmeyer flask and made up to a volume of 25 ml with a phosphate buffer solution of pH 7.2. One thousand μg of histamine was added per g of lung tissue. The weights of the pooled tissues varied from 0.6 to 1.19 g. After the addition of 5 drops of toluene, the mixture was incubated at 37°C for 16 hours. At the end of the incubation period, the sample was heated at 90°C for 3 minutes, filtered, and diluted with buffer; the histamine remaining was assayed by measuring the fall in blood pressure of an atropinized cat under barbiturate anesthesia. Cats were anesthetized by intraperitoneal injections of 30 mg/kg of sodium pentobarbital. Atropinization was accomplished with hourly subcutaneous injections of 1 mg/kg of atropine sulfate and the effectiveness of atropinization was checked every 30 minutes by the failure of 2 μg of acetylcholine injected intravenously to cause a drop in blood pressure. The fall in diastolic blood pressure due to histamine was recorded at the carotid artery by means of a Statham Transducer in conjunction with a Brush strain analyzer (Model B1-310) and oscillograph (Model B1-202). Intravenous doses of a standard histamine solution were alternated with doses of the unknown and the error of each assay was deducted from the data of the experiment itself by the method of Vos(6). The experimental error varied from 2 to 22%.

TABLE I. Inactivation of Histamine by Lung Tissue from Histamine-Sensitive and Histamine-Resistant Mice.

	Histamine-resistant mice		Histamine sensitive mice	
	Exp. No.	Histamine destroyed per g tissue in 16 hr* (μg)	Exp. No.	Histamine destroyed per g tissue in 16 hr† (μg)
T§	1	512.5	3	181.3
C§		0‡		0
T	2	582.0	4	154.7
C		0		0
T	6	503.4	5	191.0
C		0		0
T	7	522.0	9	183.3
C		0		0
T	8	571.6	10	164.4
C		0		0

* Mean ± S.E. 538.9 ± 16.2. Statistically significant ($p = <.01$) when compared with values for histamine-sensitive mice.

† Mean ± S.E. 174.9 ± 7.6.

‡ Destruction of histamine less than 5% was considered negligible.

§ T = test; C = control.

As controls, the minced lungs of 5 histamine-sensitive or 5 histamine-resistant mice were heated for 3 minutes at 90°C prior to their incubation with histamine. The control samples were then treated exactly as the test mixtures. In the case of the controls, at least 96% of the added histamine was always recovered. Unheated tissues from uninoculated mice destroyed 55 ± 3% of the added histamine; whereas, unheated tissue from pertussis-inoculated animals destroyed only 17 ± 2% of the added histamine. In all, a total of 10 experiments were carried out in which 100 mice were employed.

Results and discussion. The data in Table I indicates that lung tissue from uninoculated mice inactivated 538.9 ± 16.2 mg histamine/g tissue, as compared with 174.9 ± 7.6 mg for pertussis-inoculated mice. The difference between these values is highly significant ($p = < .01$). These observations suggest that mice inoculated with pertussis vaccine become sensitive to the lethal effects of histamine by virtue of the reduced ability of their tissues to inactivate this amine.

[†] The pertussis vaccine employed (50 billion organisms/ml) was supplied through the courtesy of Dr. W. F. Verwey, Sharpe & Dohme Inc., West Point, Pa.

H. pertussis vaccine may have a toxic effect on the enzyme, presumably histaminase, which destroys histamine. This vaccine may also exert its effect indirectly by damaging the adrenal gland. The tissues of an adrenalectomized animal have a reduced histaminase activity(7). In their sensitivity to histamine, to bacterial vaccines and to anaphylaxis, pertussis inoculated mice are very similar to adrenalectomized animals(2).

Summary. Lung tissue from pertussis-inoculated, histamine-sensitive mice has a reduced ability to destroy histamine as compared with lung tissue from uninoculated, histamine-resistant animals.

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Influence of Hypophysectomy and Growth Hormone on Cartilage Sulfate Metabolism.[*] (20726)

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The biochemical effects of growth hormone on cartilage are virtually unknown, yet the proliferative response of epiphyseal cartilage to growth hormone is one of the most impressive biological affects of this endocrine substance when administered to immature hypophysectomized rats. The recent demonstrations(1,2) of the uptake of radioactive sulfate by cartilage and its incorporation into chondroitin sulfate suggested that this process might be profitably explored as to its endocrine control. Toward this end, the effects of hypophysectomy and growth hormone injection on the uptake of radioactive sulfate have been examined relative to the normal animal and are here described.

Methods. A tracer dose of S^{35} labeled inorganic sulfate containing 1.5 x 10^7 counts per minute in a 0.1 ml of saline was injected into the saphenous vein of ether anaesthetized rats. Blood samples were removed after 13, 19 and 25 hours of equilibration and the heparinized plasma was separated by centrifugation. Plasma samples were deproteinized with an equal volume of 10% trichloracetic acid and the inorganic sulfate precipitated with an ethanolic solution of benzidine(3). The twice ethanol-washed precipitate of benzidine sulfate was then determined colorimetrically with β-napthoquinone sulfonate(4). An aliquot of the dissolved benzidine sulfate solution was plated on a copper planchet and radioactivity estimated at virtual infinite thinness. For the determination of the specific activity of cartilage sulfate (inorganic and esterified), samples of rib cartilage were dissected free of adherent tissues and 40-50 mg were digested with 3 ml 4 N HCl for 2 hours in a boiling water bath after which the samples were evaporated to dryness. Solution of the residue in 5 ml of water was almost complete and any undissolved carbonized residue was removed by centrifugation and discarded. The pH of the solution was between 2.0-2.5. The sulfate was precipitated with ethanolic benzidine, filtered on paper and air-dried. The radioactivity of the benzidine sulfate precipi-

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[*] Aided by grants from the U. S. Public Health Service.

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TABLE I. The Effect of Growth Hormone on S^{35} Specific Activity of Plasma and Cartilage.

Rat type*	Hr after S^{35} inj.	Plasma S^{35} specific activity†	Cartilage S^{35} specific activity	Cartilage specific activity‡ / Plasma specific activity
Normal	13	2.8	2.6	.93 (.6 –1.3)
Hyp		14.1	.5	.04 (.03– .04)
Hyp + GH		11.4	1.1	.10 (.08– .11)
Normal	19	1.5	2.5	1.7 (1.4 –1.8)
Hyp		9.9	.7	.07 (.04– .09)
Hyp + GH		6.5	2.3	.35 (.3 – .4)
Normal	25	1.4	2.0	1.4 (1.0 –1.8)
Hyp		4.0	.6	.15 (.1 – .2)
Hyp + GH		3.9	1.8	.46 (.4 – .6)

* 4-5 rats in each group.
† Specific activity = counts/min. × 10^4/mg sulfur.
‡ Avg value and range of variation.

tate on the filter paper was measured and suitable corrections for self-absorption applied since mass of the precipitate was appreciable. All samples were corrected for decay. The benzidine sulfate precipitate was estimated colorimetrically as in the case of plasma.

Rats of the Long-Evans strain were hypophysectomized at 26 days of age and injections with either growth hormone or saline began on day 12-13 post-operative. For comparison with untreated hypophysectomized rats, normal control rats 28 days of age were used. All animals were females and weighed 70 g with the maximum variation being ±5%. All animals were maintained on Diet 1(5) fed *ad libitum* during the experiment.

The growth hormone was prepared essentially by the Li salt fractionation method(6). The minimum effective dose for thyrotropic and interstitial-cell stimulating activities in the growth hormone preparations used was 4 mg as determined by the 4-day histological repair test using immature hypophysectomized rats. The minimum effective dose of growth hormone necessary to evoke a significant epiphyseal cartilage response in the 4-day tibia test (7) was a total dose of 10 μg. Hypophysectomized rats treated with growth hormone were given 25 μg daily for 3 days following which labeled sulfate was injected one hour after the last growth hormone injection.

Results and discussion. In Table I are summarized the plasma inorganic sulfate and cartilage inorganic and esterified sulfate specific activities after 13, 19 and 25 hours.

Hypophysectomy of the immature growing rat markedly decreases the uptake of S^{35} by costal cartilage. When the cartilage:plasma sulfate ratios of hypophysectomized and normal rats are compared, it can be seen that in the hypophysectomized rat S^{35} uptake is never more than 11% of normal. The greatly diminished uptake of the tracer by hypophysectomized rat cartilage presumably is responsible in part for the relatively smaller dilution of the injected tracer.

As it is evident from cartilage:plasma specific activity ratios shown in Table I, growth hormone has a small but nonetheless unmistakable effect in restoring the ratios toward normal. Thus, at 19 and 25 hours after injection of the tracer, the specific activity ratios in the growth hormone treated rats were 21% and 33% of normal respectively. Uptake in the untreated hypophysectomized rats was 4% and 11% at the corresponding time intervals.

The restoration of the specific activity ratio in the growth hormone hypophysectomized rat has not been attained under the present experimental conditions. It is possible that a longer period of treatment with a higher dose level of growth hormone may be required. Furthermore, the well known synergism between growth hormone and thyroxin may necessitate the combined injection of the two hormones for the complete repair of cartilage sulfate metabolism in hypophysectomized rats.

Summary. Hypophysectomy of immature growing rats results in a markedly diminished sulfate uptake of costal cartilage as judged from the cartilage to plasma inorganic sulfate specific activity ratios. The injection of

growth hormone partially restores this ratio to normal under the conditions employed. The plasma inorganic sulfate specific activities of hypophysectomized rats are several fold higher than those of normal rats.

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A Fibrinolytic System in Human Milk.*† (20727)

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The presence of an activator of plasminogen in urine suggested a search for fibrinolytic activity in other fluids secreted or excreted through narrow channels(1). Milk and casein are known to contain small amounts of a proteolytic enzyme(2). The experiments to be presented here show that there exists in human milk a fibrinolytic system resembling that demonstrated in human blood(3). This result is in accordance with the independent observation by Geiger(4), that streptokinase increases the proteolytic activity of mixtures of milk with rabbit plasma.

Materials and methods. Enzymic activity was estimated by the following 3 methods: I. Fibrinolytic effect was estimated on bovine fibrin containing plasminogen by the standard fibrin plate method(5) and recorded as the product (in mm²) of 2 diameters of the lysed zones (average of 3 determinations). II. Effect on bovine fibrin, where plasminogen had been destroyed by heating, was estimated similarly on fibrin plates heated for 45 min. at 85°C(6). III. Proteolytic activity was estimated on casein (3% in borate or phosphate buffer) after precipitation with perchloric acid (final concentration 6%) by determination of the optical density of the supernatant at 270 mμ using a Hilger Uvispek spectrophotometer.

Human milk was obtained fresh from The Children's Hospital, Fuglebakken, Copenhagen. The samples were centrifuged, and the skimmed milk was used. Bovine plasminogen was prepared from ammonium sulfate precipitated fibrinogen(5), using 0.9% NaCl instead of the usual barbital buffer) by heating for 10 minutes at 54°C followed by dialysis of the supernatant against saturated ammonium sulfate. The precipitate was redissolved, dialysed against borate buffer, and lyophilized. Streptokinase ("Varidase") was obtained from the Lederle Laboratories. Buffers: Barbital: 0.1 M; pH 7.75; NaCl to $\mu = 0.15$. Borate: 0.05 M; pH 7.75; NaCl to $\mu = 0.15$. Phosphate: 0.06 M; pH 7.35; $\mu = 0.15$.

Fibrinolytic activity of human milk. A wide range of fibrinolytic activity was encountered when samples of human milk were applied to standard fibrin plates. Activity measurements of about 400 mm² were obtained maximally and a considerable number of samples showed no or only a slight activity. In all samples of milk the addition of streptokinase produced a high fibrinolytic activity. The streptokinase solution alone had only a very slight effect on the standard fibrin plates (Fig. 1). However, no or only a trace of activity was found when the effect

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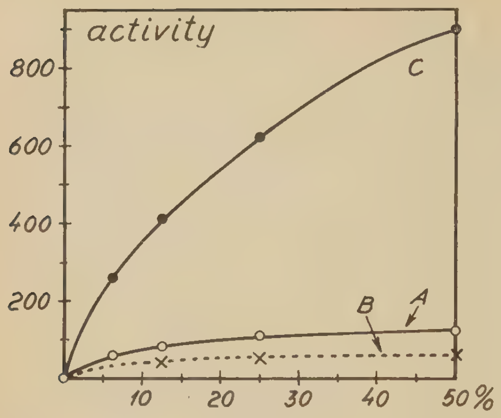

FIG. 1. Action of streptokinase on human milk. *Abscissa*: Final concentrations in % of stock solutions. *Ordinate*: Fibrinolytic activity on standard fibrin plates. *Curve A*: Serial dilutions of milk with barbital buffer. *Curve B*: Serial dilutions of streptokinase (stock solution: 1 mg in 64 ml barbital buffer). *Curve C*: Serial dilutions of human milk containing equal amounts of streptokinase (in conc. 50% of stock sol.).

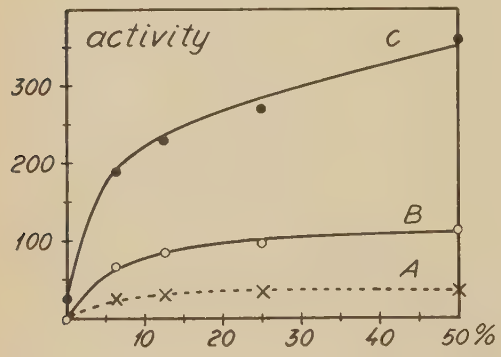

FIG. 2. Effect of plasminogen on the fibrinolytic activity of streptokinase activated human milk. *Abscissa*: Final concentration of plasminogen in % of stock solution (20 mg/ml in barbital buffer). *Ordinate*: Fibrinolytic activity on heated fibrin plates. *Curve A*: Serial dilutions (1 ml) of plasminogen with diluted (50%) streptokinase solution (1 ml; stock solution: 1 mg in 50 ml barbital buffer). *Curve B*: Serial dilutions (1 ml) of plasminogen with diluted (50%) human milk (1 ml). *Curve C*: Serial dilutions (1 ml) of plasminogen with 1 ml mixture prepared by heating human milk with equal vol of streptokinase stock solution for 30 min. at 37°C. *Controls*: Human milk, streptokinase or plasminogen alone: No activity. Mixture of milk and streptokinase: Trace of activity (25 mm²).

of the milk on heated fibrin plates was studied. This was true before as well as after the addition of streptokinase to the samples. Though a denaturation occurs during the heat treatment of the fibrin this does not significantly interfere with the lytic effect of a number of proteolytic enzymes(6). Bovine fibrin normally contains large amounts of plasminogen, but in the heated fibrin plates the plasminogen is destroyed. The fibrinolytic activity observed on standard fibrin plates is therefore probably caused by an activation of the plasminogen contained in the fibrin. This means that the fibrinolytic agent present in milk acts as an activator of plasminogen, and that the action of streptokinase might consist in the transformation of a proactivator into an activator of plasminogen. This is similar to the system found in human blood(3).

Effects of plasminogen. If a plasminogen activator is involved, the addition of plasminogen should result in the formation of plasmin. Appropriate mixtures of human milk, plasminogen and streptokinase were therefore prepared and the resulting activities measured on heated fibrin plates (Fig. 2). It is seen that streptokinase alone produced a very low activity in the bovine plasminogen. The effect produced by a mixture of human milk and bovine plasminogen was small in this experiment. This effect was found in other experiments to depend upon the fibrinolytic activity of the sample of human milk. Very large activities were always found when a mixture of human milk and streptokinase was added to plasminogen. The effects increased with increasing concentrations of plasminogen. These results therefore prove the assumption made above concerning a plasminogen activator and proactivator.

A dry preparation. An effort to control these results by estimating the proteolytic activity on casein was complicated by high blank values in the estimation of proteolytic activity by light absorption at 270 mμ. This difficulty was overcome by treating with chloroform (1 ml per 10 ml milk; 45 minutes slow shaking at room temperature), followed by isoelectric precipitation at pH 4.5 of the resulting (undiluted) solution, resolution of the precipitate in borate buffer, and lyophilization. Less than half the activity remained after these procedures.

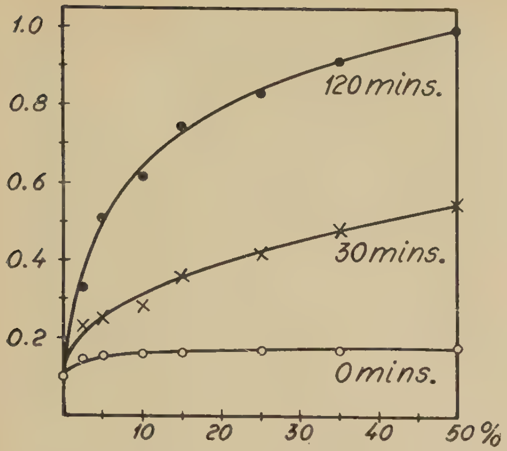

FIG. 3. Digestion of casein with plasminogen in presence of streptokinase activated human milk. *Stock solutions*: Bovine plasminogen: 30 mg/ml H_2O. Milk preparation: 25 mg/ml H_2O. Streptokinase: 1 mg in 25 ml phosphate buffer. Dilutions of plasminogen prepared in phosphate buffer: 100, 70, 50, 30, 20, 10, 5, 0%, respectively. *A*: Dilution of milk preparation: 16.5 ml stock sol. + 5.5 ml phosphate buffer. *B*: Dilution of streptokinase: 5.5 ml stock solution + 16.5 ml phosphate buffer. *C*: Mixture of milk preparation (16.5 ml) with streptokinase solution (5.5 ml). Samples (2.5) of plasminogen solutions mixed with 2.5 ml of A, B, and C. After 30 min. at 37° 4.5 ml were added to 4.5 ml casein solution. Samples of 2 ml removed after 0, 30 and 120 min. and added to 3 ml 10% perchloric acid. Samples were left overnight, centrifuged, and optical density at 270 mμ estimated. *Abscissa*: Final concentration of plasminogen in % of stock sol. *Ordinate*: Optical density at 270 mμ. Curves for casein digestion at zero time, after 30 and 120 min. (Series C). Controls (optical density): Casein alone (max 0.060). Streptokinase + plasminogen + casein (max after 2 hr: 0.092) (Series B). Milk preparation + plasminogen + casein (max after 2 hr: 0.116) (Series A).

Casein digestion. By means of this partially purified dry preparation it was possible now to confirm on a casein substrate the existence of the plasminogen proactivator demonstrated in the fibrin plate experiments. Appropriate mixtures of the partially purified milk agent with streptokinase were incubated with plasminogen for 30 minutes and the solutions then added to the casein solution. After digestion at 37°C for either 30 minutes or 2 hours, samples were precipitated with perchloric acid solution; and the optical density of the supernatant was read at 270 mμ. For details see Fig. 3. The results compare exactly with those obtained with streptokinase on the heated fibrin plates.

Discussion. The results presented here show that human milk contains: 1. An agent which acts as an activator of plasminogen, and which is present in varying and usually slight amounts. This agent was also recently found in bovine milk. 2. An agent which in the presence of streptokinase is transformed into an activator of plasminogen. This agent is present in all samples in considerable amounts.

The existence of a fibrinolytic system in milk is of interest because it supports the idea(1) that fluids occurring in narrow channels in the organism are provided with mechanisms by which the obstruction of the channels with clotted fibrin can be counteracted. The similarity of this system with that found in blood(3) suggests that the origin of these substances in milk is probably to be found in the blood. The observations are therefore also of significance in the discussion of the blood proteins as a source of some of the proteins contained in milk. However, a simple nonselective contamination of the milk with blood proteins appears less probable because other experiments showed, that human milk contains very small amounts of the trypsin inhibitor which is present in high concentration in blood. In this respect it is of less significance that the amount of plasminogen in human milk was found to be so small that it could hardly be observed by the methods applied. Since the plasminogen content of human blood is low(3), dilution might make it difficult to detect the small amounts present.

It is worth noticing that the chloroform treatment which is used in producing fibrinolytic activity in blood(7), does not produce fibrinolytic activity in milk. However, the development of activity in blood after short treatments was very slow(8).

The relation between the activities observed here and the slight proteolytic activity found in milk and casein by previous authors (2) is not known.

Summary. 1. Human milk contains varying amounts of an agent, which activates plasminogen to plasmin. 2. Human milk contains considerable amounts of a proactivator, which

by addition of streptokinase is transformed into an activator of plasminogen.

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Observations on the Nature of Radioactive Contaminants in Biosynthetic Cholesterol-C^{14}.* (20728)

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Schwenk and co-workers have reported that the digitonide of biosynthetic cholesterol-C^{14} is contaminated in certain instances by unidentified compounds of high specific radioactivity(1-3). This phenomenon has been of particular interest because of the possibility that the contaminating substances might represent biological precursors of cholesterol.

It has been found in this laboratory that up to 80% of the radioactivity of the sterol digitonide obtained from the livers of rats sacrificed 3 minutes after injection of sodium acetate-1-C^{14} was due to non-cholesterol contaminants. A number of substances related either chemically or biologically to cholesterol have been examined as possible sources of radioactive contamination.

Experimental. Each experiment employed cholesterol digitonide obtained from the pooled livers of several animals. Normal adult male Wistar rats were anesthetized by intraperitoneal injection of sodium pentobarbital, 6 mg per 100 g body weight. After exposure of the femoral vein, each animal was given 4.0-6.7 mg of sodium acetate-1-C^{14} intravenously (total radioactivity 5.8-13.4 x 10^6 counts/min). Three minutes later the liver was rapidly excised and immersed into 10 volumes of hot 95% ethanol containing 12% KOH. The mixture was boiled for 3 hours under reflux and filtered through sintered glass. Sufficient water was added to the filtrate to reduce the ethanol concentration to 50%, and the non-saponifiable material was obtained by 3 extractions with one-half volume of petroleum ether (B.P. 60-68°). The petroleum ether extracts were combined, washed once with 5% sodium acetate and twice with water, then dried over Na_2SO_4 and evaporated to dryness under N_2. The residue was taken up in 1:1 acetone-ethanol, and cholesterol digitonide was precipitated by the addition of a 1% solution of digitonin in 80% ethanol. The digitonide was washed twice with 80% ethanol and once with ether.

In a preliminary experiment sodium acetate-1-C^{14} containing 2.5 x 10^5 counts/min was added to the liver of a normal rat, and cholesterol digitonide isolated as described above. The digitonide was free of radioactivity. Radioactivity assay was carried out with a windowless flow gas counter. Cholesterol, 3β-cholestanol and 7-ketocholesterol were counted as the digitonides, mounted on sintered alundum discs. Other compounds were subjected to wet combustion by a modification of the method of Lindenbaum *et al.*(4) and counted as $BaCO_3$ mounted on alundum discs.

Exp. 1. The digitonide obtained in one ex-

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† Postdoctoral Fellow of the Life Insurance Medical Research Fund.

‡ Fellow of the Public Health Research Institute of the City of New York, Inc.

periment had a specific radioactivity of 39 counts/min/μmole. The free sterol was prepared from this digitonide with pyridine and ether. Cholesterol isolated via the dibromide (1) from a portion of this crude sterol had a specific activity of only 12 counts/min/μmole. 5.7 mg of unlabeled 7-ketocholesterol and 9.0 mg of unlabeled 7β-hydroxycholesterol were added to 8.5 mg of the crude sterol. This mixture was separated by column partition chromatography, using the solvent system propylene glycol-petroleum ether(5). The digitonide prepared from the cholesterol band had a specific activity of 33 counts/min/μmole; both the 7-ketocholesterol and 7β-hydroxycholesterol bands were non-radioactive within the precision of measurement. Another portion (6.5 mg) of the crude sterol was chromatographed with 7.9 mg of cholestane-3β,5α,6β-triol on a partition column using the solvent system aqueous methanol-cyclohexane(5). The digitonide prepared from the cholesterol band had 34 counts/min/μmole, while the triol was non-radioactive. A mixture of 3 mg of unlabeled cholesterol and 0.5 mg of acetic acid-1-C^{14}, containing 1 x 10^6 counts/min, was chromatographed using the system propylene glycol-petroleum ether. The eluted cholesterol was free of radioactivity.

Exp. 2. Another sample of "contaminated" cholesterol digitonide had a specific activity of 163 counts/min/μmole. 50 mg of free sterol obtained from this digitonide were brominated; the 5,6-dibromocholestan-3β-ol had a specific activity of 35 counts/min/μmole. After debromination, the specific activity was 33 counts/min/μmole. To the mother liquor from the bromination were added 2.45 mg of non-radioactive 3β-cholestanol, and the digitonide of the saturated sterol fraction was precipitated. 11.9 mg of digitonide with a specific activity of 298 counts/min/μmole were obtained. It was calculated that all but 1.7 mg of this was inactive carrier digitonide, and that the activity of carrier-free sterol was approximately 2,100 counts/min/μmole. After adding unlabeled 3β-cholestanol digitonide as additional carrier, the free sterol was regenerated and isolated. The specific activity was 16 counts/min/μmole, a value consistent with the dilution by carrier. After 3 crystallizations from methanol, the specific activity fell to 1.4 counts/min/μmole. After 9 recrystallizations the 3β-cholestanol was free of detectable radioactivity.

Exp. 3. Another portion of the "contaminated" cholesterol with specific radioactivity of 163 counts/min/μmole was subjected to reversed-phase partition chromatography(6) employing heptane as the stationary phase and aqueous methanol as the mobile phase. Elutions were made with 90, 92.5, 95 and 100% methanol. Cholesterol was eluted with 92.5% methanol; the digitonide prepared from this fraction had an activity of 93 counts/min/μmole. The remainder of the radioactivity was found in fractions of negligible weight appearing just before and just after the cholesterol band. With this solvent system, squalene was eluted with 100% methanol; this fraction was free of radioactivity.

Summary. Experiments have been carried out on the nature of the radioactive contaminants in biosynthetic cholesterol-C^{14} obtained from rat livers, employing preparations in which as much as 80% of the radioactivity was due to contaminating material. At least one major contaminating substance was still precipitable with digitonin after treatment with bromine, but it was not 3β-cholestanol. The data make it unlikely that 7-ketocholesterol, 7β-hydroxycholesterol, cholestane-3β,5α,6β-triol, squalene or unutilized acetate contributed significantly to the radioactive contamination found in these preparations.

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Metabolism of Cr^{51} by Animals as Influenced by Chemical State.[*] (20729)

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The knowledge of the factors governing the physiological behavior of a radioisotope in the body may extend the base of its usefulness. Furthermore, such data are needed for the establishment of reasonable maximum permissible levels. Chromium 51 has been used satisfactorily as a tag for plasma proteins or erythrocytes depending upon the valence state (1). As far as is known, only one report on the behavior of this radioisotope in the animal body has appeared in the literature. Kraintz and Talmage(2) have reported the distribution of Cr^{51} in the tissues of 180-200 g rats for intervals up to 24 hours following the intravenous injection of the tracer in isotonic acetate buffer.

Reported in the present paper are studies on the physiological characteristics of this radioisotope over longer time periods as affected by chemical form and route of administration. Data are summarized from more than 200 rats on the uptake, excretion, retention, placental transfer, and tissue distribution of Cr^{51}. The isotope was given in the trivalent state as chloride and chromite and in solutions buffered by acetate and citrate. Hexavalent chromium was given as a solution of sodium chromate. The primary route of administration was intravenous but results are also reported for oral, intraperitoneal and intratracheal administration. In addition, data are presented from two sheep which were intravenously injected with $Cr^{51}Cl_3$. An attempt has been made to correlate the physiological findings with filter paper electrophoretic studies of the dosing solutions.

Methods. Rats of the Rockland and Carworth strains fed commercial rations were used. The age ranged from 7 to 10 months and the minimum weight was 320 g for males and 220 g for females. Solutions for administration to the animals were prepared from a stock of $Cr^{51}Cl_3$ which ranged in specific activity from 200 to 1250 uc per mg. Adjustment of acid concentrations was always made immediately prior to dosing. The chloride, chromite and chromate were given at pH 3 to 4, 11 and 7 respectively and buffered solutions of the isotope were prepared by addition of the $Cr^{51}Cl_3$ to acetate and citrate buffers calculated to be isotonic with physiological saline. For acetate the approximate pH was 5.5 and for citrate 4.0. About 50 microcuries of activity in 0.1 to 0.2 ml of solution was the dose per rat for intravenous, intraperitoneal and intratracheal administration, whereas 5 to 10 times these amounts were used for the oral studies. Intravenous injection was made into the femoral vein, and intratracheal administration was accomplished by exposure of the trachea through a ventral midline incision which allowed insertion of an 18 gauge needle and the deposition of the solution directly into the lungs. Both the latter operations were carried out under surgical anesthesia using nembutal and ether. At the time of administration, diluted reference standards were prepared from the dosing solution. The rats were sacrificed by exsanguination under anesthesia at intervals up to 6 weeks after dosing. Representative tissue samples were placed in tared nickel plated steel planchets and weighed immediately after removal from the carcasses to minimize errors due to dehydration. In all of the intravenously injected animals the site of injection was sampled as a check for quantitative administration. The tissue samples were kept in a frozen state until radioactivity was determined by direct measurement with a scintillation counter. Quantitative fecal and urine collections were made in metabolism cages described by Hansard *et al.*(3). Aliquots of urine were counted directly whereas fecal radioactivity was measured on an aliquot of the ash solution. An additional experiment was carried out with

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TABLE I. Summary of Behavior of Cr^{51} in Rats Following Intravenous Administration in Different Chemical Forms (Tissue Values Represent % of Dose per g Fresh Tissue ± Standard Error; 4 Rats per Value).

	$Na_3Cr^{51}O_2$	$Cr^{51}Cl_3$†	$Na_2Cr^{51}O_4$	$Cr^{51}Cl_3$ buffered by acetate	$Cr^{51}Cl_3$ buffered by citrate
% excreted in urine 4d*	.6 ± .12	15 ±1.3	35 ±1.9	56 ±5.1	75.1 ±9.3
% excreted in feces 4d*	1.6 ± .22	20 ±1.5	17 ±1.6	8 ±3.7	17.4 ±4.8
Liver 4d	9.5 ± .12	5.5 ± .69	.52± .06	.16 ± .01	.31 ± .12
" 42d	6.4 ± .39	2.6 ± .22	.07± .00	.19 ± .01	.17 ± .01
Spleen 4d	17 ±3.9	3.2 ± .35	.91± .16	.30 ± .03	.76 ± .28
" 42d	8.3 ± .61	4.3 ± .45	4.8 ±1.6	.41 ± .05	.23 ± .04
Bone marrow 4d	6.4 ± .84	1.5 ± .23	.56± .14	.17 ± .13	.13 ± .08
" " 42d	.94± .36	1.5 ± .28	.16± .16	<.02	<.02
Tibia epiphysis 4d	1.5 ± .33	1.09± .19	.38± .03	1.0 ± .17	.20 ± .06
" " 42d	.22± .08	.63± .05	<.02	.97 ± .12	.35 ± .06
Lungs 4d	1.6 ± .24	.24± .02	.39± .26	.11 ± .01	.21 ± .14
" 42d	.15± .01	.11± .03	.05± .01	.08 ± .01	<.02
Kidneys 4d	.06± .01	.44± .02	2.1 ±1.1	.62 ± .06	.13 ± .07
" 42d	.04± .00	.26± .03	.24± .05	.25 ± .03	.07 ± .00
Blood 4d	<.02	.05±0	.52± .07	.03 ± .01	<.02
" 42d	<.02	<.02	.04± .01	<.02	<.02
Behavior	Colloidal	Colloidal under these conditions	Probably ionic under these conditions	Complex	Complex
Electrophoretic pattern	Colloidal in serum	Protein bound	Ionic in serum bound by hemoglobin in cells	"	

* Avg of 8 animals ± stand. error.

† Animals receiving $Cr^{51}Cl_3$ were sacrificed at 45 days instead of 42.

two sheep on a normal ration of hay and grain for purposes of comparison with laboratory animals. The activity in the form of $Cr^{51}Cl_3$ was administered by jugular catheter (1 mc per 100 lbs of body weight) and the metabolism procedures were those described by Hansard *et al.*(4).

Results. Table I summarizes the distribution of radioactivity in the tissues and excreta of rats following intravenous injection of Cr^{51} in various chemical forms. The data presented show the Cr^{51} excretion and tissue concentration at 4 days and the tissue concentration of 42 days after dosing. Animals were sacrificed at six hours after dosing and at various intervals thereafter for 42 days. Only the 4- and 42-day values are reported for the liver, spleen, bone marrow, lung, kidney, tibia epiphysis and blood. With the exception of the value for the pancreas following chromate injection, all preparations of radiochromium resulted in concentration values for the pancreas, tibia shaft, incisors and mandible plus teeth which were about 50 percent of the tibial epiphysis value. For chromate the concentration in the pancreas was equal to that in the epiphysis of the tibia.

In preliminary experiments involving intravenous injections of $Cr^{51}Cl_3$ the following were found to contain insignificant concentrations of radioactivity: small intestine, large intestine, hide and hair, brain, eyes, skeletal muscle, ovaries, prostate gland, uterus and testes.

Sodium chromite. It is clearly evident that the Cr^{51} from sodium chromite was picked up in extremely large quantities by the organs of the reticulo-endothelial system and the liver accumulated approximately 90% of the dose. The excretion values were correspondingly low. A summation of the excretion values and the organ values gave recovery of the total dose within ±10%. Considering the extremely small amount excreted, and high liver and spleen values at 21 and 42 days after injection, it may be considered that 100% of the dose was held in the body during the early times after injection. The tibial epiphysis showed a higher concentration of Cr^{51} than did the other skeletal tissues, and the activity was slow in leaving all of the organs except the blood and lungs. The liver and spleen contained 33 and 50% of their 4-day activity at the end of 42 days, respectively, whereas the lung showed only 10% of its 4-day activity. No detectable radioactivity was found in the blood after 21 days.

Chromic chloride. The radioisotope was found to be in highest concentration in the liver, spleen, and bone marrow, undoubtedly a reflection of reticulo-endothelial pickup but not as great as with sodium chromite. Although there was considerable variation between animals, it is obvious that the activity once deposited was removed very slowly. The value for the liver at 45 days was 35% of its maximum value which was observed at 24 hours after dosing. In contrast, the concentration in all of the other tissues remained practically constant throughout the 45-day period with the exception of the blood and lungs. The blood contained practically no activity after the seventh day and the lungs at 45 days contained about 15% of their maximum value which was observed at 6 hours following injection. The skeletal tissues showed practically equal amounts of activity in the tibial shaft, mandible plus teeth and incisors with approximately twice as much in the epiphysis of the tibia. Interestingly, the spleen appeared to gain activity over the 42-day period. With $Cr^{51}Cl_3$ other routes of administration were studied, but only the overall results are presented. Seven days after intratracheal administration it was found that 55% of Cr^{51} was excreted in the feces and 7% in the urine which indicated that the material probably had been transported from the region of deposition in the lungs to the oral cavity where swallowing took place and subsequently the isotope was handled as after ingestion. Tissue concentrations indicated that probably less than 5 percent was absorbed from the lungs. The pattern of distribution in the tissues was more like that of the buffered Cr^{51} solutions. Orally administered $Cr^{51}Cl_3$ was almost totally excreted in the feces at the end of 4 days. Less than 0.5% of the dose was absorbed from the gastrointestinal tract as indicated by tissue distribution studies. Although the urinary excretion indicated a higher level of absorption, the urine radioactivity was probably due to fecal contamination. After intraperitoneal injections of $Cr^{51}Cl_3$, extensive necrosis developed at the site of injection due to the high acidity of the solution and the tissue uptake was variable and extremely small.

Sodium chromate. The major uptake was by the liver but in much lower amounts than for the chromic chloride or sodium chromite and the excretions were correspondingly higher. Twenty-five percent of the injected activity became deposited in the liver in 30 minutes after administration and at the end of 42 days less than one percent was present, which again represented a marked difference in behavior from the chromite and chloride. All of the other organs and tissues lost activity with time except the spleen which seemed to gain. The skeletal tissues showed relatively small concentrations of Cr^{51} and the tibia epiphysis contained twice the concentration of the other skeletal tissues sampled during the earlier time periods. Interestingly, the concentration in blood at the end of 21 days was about one-fifth of the 30-minute value but declined to practically zero at 42 days. This greater retention by blood of hexavalent Cr^{51} versus trivalent Cr^{51} was probably because the former is bound by erythrocytes which turnover relatively slowly and the latter by plasma proteins. The chromate solution, however, is the only one which gave evidence of blood radioactivity at the end of 42 days.

Chromic chloride in acetate and citrate buffers. The results following intravenous injection of $Cr^{51}Cl_3$ when isotonically buffered by acetate (pH 5.5) and citrate (pH 4) followed the same general pattern. The organ of greatest accumulation was the liver for both solutions. On a concentration basis, however, there seemed to be a difference between the 2 solutions, the epiphysis of the tibia showing the highest accumulation for acetate with the spleen showing the highest for citrate. The concentrations in all of the tissues and organs except for the lungs and blood tended to remain constant throughout 21 days and appeared to have declined in some of the other tissues by the end of 42 days. The blood contained no detectable activity at the end of 21 days. In the case of citrate, all of the bones

TABLE II. Distribution of Cr^{51} in Sheep Following Intravenous Injection of Cr^{51}Cl$_3$.

Tissue	% of dose per g fresh tissue ×10^4 Sheep 107	Sheep 68
Blood	16.6	7.5
Lung	114.	727.
Liver	78.8	28.4
Spleen	73.9	33.2
Bone marrow	42.0	25.6
Kidney	55.7	43.4
Lymph node	40.8	20.9
Pancreas	6.7	3.3
Loin muscle	2.6	.61
Gastrocnemius muscle	1.1	.4
Vertebra	27.6	29.4
Metatarsus shaft	4.3	5.2
Metatarsus epiphysis	9.2	9.5
Incisors	8.1	9.4
Angle mandible	33.3	32.5
White bone marrow	3.8	1.7
Excretions (total % of dose in 7 days)		
Urine	32.7	60.3
Feces	3.3	2.2

were about equal in Cr^{51} concentration whereas with acetate, there was a higher deposition in the tibial epiphysis than in any other tissue. The lower uptake by the organs of the reticulo-endothelial system in the case of these 2 solutions is probably the result of relative absence of large particles. Of special interest was the large urinary excretion of the activity when administered intravenously in the buffered solutions. Fifty-six percent of the acetate buffered Cr^{51} was excreted in the urine in 4 days and 8% in the feces. Respective values for the citrate buffered solution were 80% and 13%.

Placental transfer. Insignificant amounts of Cr^{51} were found to cross the placenta of rats in the 24 hours following intravenous injection regardless of the chemical form of the isotope, its valence state, the gestational stage of (15 to 20 days) the animal or the number of fetuses per litter. In no case did the recovery of Cr^{51} per total litter exceed 0.13% of the injected dose. In all of the determinations, radioactivity was measured only on the fetuses exclusive of the placental membranes or fluids.

Chromic chloride in sheep. Sheep 107, weighing 92 lb and Sheep 68, weighing 120 lb, both 5-year-old ewes, were dosed intravenously by jugular catheter with Cr^{51}Cl$_3$ and sacrificed 7 days later for tissue distribution studies. During the 7-day interim all excretions were collected for radioassay. The results are presented in Table II. There was considerable variation in the results between the 2 animals which may have been caused by a difference in the mass of chromium contained in the 2 doses since Sheep 107 received 4 mg of chromium, whereas Sheep 68 received 8 mg. Though, in preliminary experiments, the mass of chromium injected was found to affect the uptake of Cr^{51} by the lungs of rats, it is questionable whether the differences in the dosage masses of the sheep were sufficient to cause the differences in lung uptake observed. Both sheep showed maximum retention in the lungs with the liver being next highest. The pattern of tissue distribution was quite similar for both animals although some of the actual values were not in close agreement. Sheep 107 deposited 7% of the activity in her lungs and 5% in the liver, whereas the respective values for Sheep 68 were 33% and 2%. The spleen and bone marrow showed appreciable concentrations of Cr^{51} and the lymph nodes fell in a range comparable to the bone marrow, vertebrae and angle of the mandible. The white bone marrow was extremely low in activity in contrast to the red bone marrow. Of the skeletal tissues studied, the vertebrae and angle of mandible were highest and the tibia shaft and epiphysis and incisors were very low. All of the muscle tissues sampled were extremely low in activity. The excretions also showed considerable variation between animals with Sheep 107 excreting 33% in the urine and 3% in the feces in 7 days. Respective values for Sheep 68 were 60.0 and 2.2. Over 80% of the 7-day urinary excretion took place on the first day after injection.

Paper electrophoretic studies on various Cr^{51} solutions. Paper electrophoresis was performed with the apparatus designed by Gordon *et al.*(5) using 0.05 Na barbital or homologous serum as a buffer. The procedure followed and a more complete report of the findings are included in another publication (6). Sodium chromite incubated with rat serum and applied to paper strips buffered by the homologous serum was found to lack mobility which supported the view that the chromite was colloidal in this medium. This evidence led to some limited studies in which sodium chromite was incubated with rat serum and intravenously injected. A high uptake by the reticulo-endothelial system resulted which strengthened the view that chromite behaved as a colloid *in vivo*. Chromic chloride incubated in rat serum and applied either to a barbiturate or serum buffered strip moved predominantly with the serum protein indicating protein binding. These results were surprising and led us to intravenous injection of the incubated mixture which resulted in less than 10% of the activity being taken up by the liver in contradistinction to approximately 50% when the solution was injected directly into the blood stream. Apparently direct injection of the solution caused the formation of particles which were phagocytized before protein binding could take place. When chromic chloride was complexed with acetate before incubation with rat serum the resulting electrophoretic patterns did not show appreciable protein binding. Sodium chromate incubated for 16 hours with whole rat blood showed the activity in the red cells to be bound to the hemoglobin fraction. Chromate was found to move as a free ion after incubation in serum.

Discussion. These experiments emphasize the variations in physiological behavior of chromium which may be brought about by its physico-chemical state. The behavior of radiochromium varied as a function of chemical form prior to injection, the solvent used and the route of administration. These factors assume great importance with elements that are prone to form colloids as pointed out by Schubert(7). Differences in the physiological behavior of the chromium in the animal resulting from the various solutions were in fair correlation with the predictions one might make from the paper electrophoretic patterns observed in serum and lysed erythrocytes *in vitro*(7-11).

Trivalent Cr^{51} might be expected to differ in its physiological behavior from the hexavalent form since the former is bound by plasma proteins and the latter by erythrocytes(1). However, the variations in the behavior of trivalent chromium require further explanation. The trivalent chromium, namely chromite, chromic chloride, and buffered chromic chloride showed respectively an increasing excretion and a decreasing pickup by the reticulo-endothelial system. This behavior may be explained on the basis of the colloidal nature of the chromite, the protein binding of the chromic chloride, and the complexing action of the buffers. Since acetate and citrate apparently form complex ions with chromium there is a possibility that acetate and citrate compounds might be used as therapeutic agents to increase the urinary elimination of Cr^{51} from the body as has been done with plutonium(7). The preparation showing greatest deposition in the reticulo-endothelial system was the chromite which apparently formed large particles as shown by paper electrophoresis, and for this reason would be expected to be so distributed *in vivo*(7-11). The liver did not always show the highest concentration of Cr^{51}. In the case of the acetate buffered Cr^{51} the epiphysis of the tibia showed the highest concentration. Thus, an indication is given as to the manner in which the localization of Cr^{51} may be governed by the chemical form used. For example, if a high liver concentration were desired, sodium chromite would be the logical compound to use. On the other hand, a buffered chromic chloride solution, or chromic chloride after incubation with serum would yield a more generalized distribution. Of particular importance from a health physics viewpoint is the observation that once the radiochromium was deposited in an organ it was slow in leaving. The fact that Cr^{51}Cl$_3$ incubated with serum showed a much lower reticulo-endothelial uptake after injection as compared to that of the solution itself points out the effects which form of injection may have on the experimental results.

Kraintz and Talmage(2) observed an apparent species difference between rats and rabbits in the behavior of Cr^{51} buffered with acetate. In their studies the rat showed the highest concentration of Cr^{51} in the bone at 24 hours, whereas in rabbits the spleen was highest. In our studies with rats, the organ of maximum uptake following intravenous injection of acetate buffered $Cr^{51}Cl_3$ was also the bone. With $Cr^{51}Cl_3$ in rats and sheep the organs of maximum concentration were the liver and lung, respectively. The high uptake by the lung of the sheep may have resulted from increased phagocytosis of particulate material by the lungs of these animals due to a lung infection which had been prevalent in the flock.

Summary. The physiological behavior of trivalent and hexavalent Cr^{51} has been observed over a 45-day period in rats as affected by chemical form and route of administration. When intravenously administered as sodium chromite, practically 100% of the dose was picked up by the organs of the reticulo-endothelial system. Chromic chloride resulted in a pickup by the liver of about 55% of the dose. When $Cr^{51}Cl_3$ was added to isotonic acetate and citrate buffers, less than 5% of the dose reached the liver and most of the activity was excreted in the urine. The use of sodium chromate solutions resulted in about 25% of the dose reaching the liver. However, the activity left this organ rapidly in contradistinction to the other forms studied. Oral, intraperitoneal and intratracheal administration of $Cr^{51}Cl_3$ were also studied. None of the forms of Cr^{51} used were found to cross the placenta of rats ranging from 15 to 20 days in gestation. In addition, data are presented from 2 sheep intravenously dosed with $Cr^{51}Cl_3$. The above physiological observations are correlated with the colloidal behavior, complex formation, protein binding, and red cell binding, and are in general agreement with interpretations of filter paper electrophoretic studies of the dosing solutions.

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Decreased Resistance of Vitamin B_{12}-Deficient Rats to Cold Stress.* (20730)

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Considerable data are available indicating that requirements for a number of nutrients are increased and resistance to cold stress is decreased in the nutritionally-deficient animal. In the present communication data are presented on 1) the effects of prolonged exposure to cold on the vit. B_{12} requirement of the rat and 2) the effects of vit. B_{12} deficiency on resistance to cold stress in the rat.

Procedure. The basal ration employed in the present experiment consisted of expeller-processed soybean flour,† 66%; sucrose,

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† Soy Flour Lo-Fat, A. E. Staley Mfg. Co., Decatur, Ill.

24.4%; salt mixture,‡ 4%; cottonseed oil (Wesson), 3%; wheat germ oil (VioBin), 2%; and dl-methionine, 0.6%. To each kg of the above diet were added the following synthetic vitamins: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 2 mg; 2-methylnaphthoquinone, 5 mg; folic acid, 10 mg; ascorbic acid, 200 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; vit. B_{12}, 100 μg; and choline chloride, 2 g. To each kg of diet were also added 8000 U.S.P. units of vit. A§ and 800 U.S.P. units of vit. D.‖ The vitamins were added in place of an equal amount of sucrose. Tests were conducted 1) with rats fed the basal ration and 2) with animals fed a similar diet with vit. B_{12} omitted.

Results. Exp. 1. Seventy female rats of the Long-Evans strain were selected at 25 to 28 days of age and at a body weight of 52 to 60 g and were fed the diets indicated above for 12 weeks or until death, whichever occurred sooner. Tests were conducted 1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2 ± 1.5°C and 2) under standard laboratory conditions at an average temperature of approximately 23 ± 2°C. Rats were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib.* (20 rats per group in the cold room series; and 12 and 18 rats per group on the basal ration and deficient diet respectively in the room temperature series). Diets were made up weekly and stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet.

Findings indicate that under room temperature conditions all rats tested survived the 12-week feeding period. The average gain in body weight of rats on the basal ration during this period was 140.8 ± 3.8 g,¶ and the average weight increment of rats fed the vit. B_{12}-deficient diet was 126.9 ± 5.3 g.¶ The difference in weight increment between these 2 groups was not statistically significant.** In the cold room series gain in body weight was retarded in all rats. Fifteen out of 20 (75%) of the rats fed the basal ration survived the 12-week experimental period with a weight increment of 93.5 ± 4.9 g;¶ 14 out of 20 (70%) fed the vit. B_{12}-deficient diet also survived with a weight increment of 69.8 ± 3.0 g.¶ Gain in body weight of rats fed the basal ration was statistically significantly greater than that of rats fed the vit. B_{12}-deficient diet. Grossly no significant differences were observed between rats in the various groups. Since the weight increment of rats fed the vit. B_{12}-containing ration was significantly greater under cold room conditions than that of rats fed a similar diet with vit. B_{12} omitted, whereas no significant difference in weight increment was observed in rats fed these diets under room temperature conditions, it would appear that requirements for vit. B_{12} were increased by exposure to low environmental temperature under conditions of the present experiment.

Exp. 2. Following the 12-week feeding period in Exp. 1, animals on both diets in the room temperature series were bred to normal males. Nine litters were cast by rats on the vit. B_{12}-deficient diet of which 3 were weaned at 21 days of age at an average weight of 32.4 g per rat. Six of the litters and 2 of the mothers succumbed during the lactation period. Twelve litters were cast by rats on the basal ration of which 6 were weaned at 21 days of age at an average weight of 35.2 g per rat. The remaining 6 litters died during the lactation period. Following weaning the young in each group were continued on the same diet for an additional 30 days or until death, whichever occurred sooner. In agreement with earlier findings(1) a number of the young on the vit. B_{12}-deficient diet (5 out of 18) died within 3 weeks after weaning. Six male rats and 7 female rats, however, survived the 30-day post-weaning period with an average increment in body weight of 38.9 ± 4.4 g¶ and 32.3 ± 3.4 g,¶ respectively. In contrast all the young on the basal ration (17 males and 20 females) survived the 30-day post-weaning period with an average gain in body weight of 98.7 ± 6.5 g,¶ and 75.4 ± 2.6 g¶ respectively. At this time the surviving rats in the vit. B_{12}-deficient group and 10 male and 10 female rats of the group fed the basal ration were placed in a cold room maintained at a temperature of 2 ± 1.5°C. No change was made in the diets fed. Feeding was continued for an additional 21 days or until death, whichever occurred sooner. All rats fed the vit. B_{12}-deficient diet died during the first 96 hours of cold exposure with over half the animals succumbing within the first 18 hours. In contrast, all rats fed the basal ration survived the 3-week period of cold exposure.

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‡ Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Crystalets, Chas. Pfizer and Co., New York, N. Y.

‖ HY-DEE Powder, Standard Brands, New York, N. Y.

¶ Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

** The failure of rats fed the vit. B_{12}-deficient diet to exhibit growth retardation under room temperature conditions was probably due to pre-test storage of vit. B_{12} during the period of pregnancy and lactation(12). That this diet was actually deficient in vit. B_{12} was demonstrated by the growth retardation and post-weaning deaths of the young born to mothers on the depletion diet in *exp. 2* and the absence of these disorders in rats fed a similar diet supplemented with crystalline vit. B_{12}.

Discussion. Available data indicate that requirements for a number of nutrients are increased under conditions of low environmental temperature. This is particularly true for some of the water-soluble vitamins. An increased requirement for thiamine(2,3), riboflavin(4), pyridoxine(5), and ascorbic acid (6) has been demonstrated following prolonged exposure to cold. In addition resistance to cold stress was significantly impaired in rats deficient in pyridoxine(7), riboflavin (8), pantothenic acid(9), and vit. A(10,11), or fed restricted amounts of a complete ration(9). It is becoming increasingly apparent that the inability of deficient rats to withstand sudden exposure to cold is due to an impaired adjustment to this type of stressor agent and not to an increased nutritional requirement and the consequent accentuation of the deficiency state. Present findings indicate that requirements for vit. B_{12} are similarly increased under conditions of low environmental temperature and that rats deficient in vit. B_{12} also have a decreased resistance to cold.

Summary. 1. Prolonged exposure to cold significantly increased the vit. B_{12} requirement of the rat. 2. Resistance to cold stress (as measured by length of survival under conditions of low environmental temperature) was significantly impaired in the vit. B_{12}-deficient rat.

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Reduction in Plasma Desoxyribonuclease Activity on Circulation through the Liver: Index of Liver Function.* (20731)

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In a recent report(1), reference was made to our observation that serum desoxyribonuclease (DNase) is regularly considerably elevated in parenchymatous liver disease but not in obstructive icterus in man. The following preliminary report is based upon studies to elucidate the role of the liver in serum DNase metabolism.

Methods. Serum DNase activity was measured at pH 7.5 (trismethylolamino-methane buffer) by our methyl green method(2). This is approximately the optimum pH for serum DNase in the rat, and no other optima are found between pH 3.0 and 9.5(3). Wistar albino rats weighing approximately 250 g (except where the weight is noted in the table) were used. All were fed a Purina Dog Chow Checkers diet and water *ad lib* up to the time of sacrificing. Under sodium pentobarbital anesthesia (approximately 3 mg/100 g; in some experiments ether alone or ether supplementation of pentobarbital was used), the abdomen was opened widely. By separating the lobes of the liver, a large hepatic vein draining the left lobe is exposed. From this hepatic vein approximately 2 ml of blood were drawn into a syringe moistened with heparin (Heparin sodium, Upjohn, 10 mg/ml). A hemostat was then applied to the portal vein close to the liver and 1.5 to 2 ml of blood were drawn into a heparinized syringe from the portal vein. The plasma was promptly separated by centrifugation, and sodium ethyl mercuri-thiosalicylate (Merthiolate, Lilly) added to a final concentration of 1:100,000. The plasma was kept frozen (–20°C) until used. One-half ml was used for the DNase analysis. In several cases blood was also collected from the abdominal aorta and similarly treated. Since the rat has no gall bladder, it is necessary to cannulate the common bile duct to collect bile. A No. 22 stainless steel cannula was used. The duct was cannulated just below the liver so as to exclude the pancreatic secretion, which enters the lower four-fifths of the bile duct(4). Approximately 1 ml of bile can be collected over a 3-hour period. The DNase activity was determined as for serum.

Several rats were given a single intraperitoneal injection of 0.05 ml carbon tetrachloride per 100 g body weight in 1 ml sesame oil 24 hours or 48 hours before sacrificing. Another group was rendered anoxic under anesthesia by respiring air bubbled through alkaline pyrogallate solution through a tracheotomy. Anoxia was maintained for several minutes after the organs, including the liver, had become intensely cyanotic. Blood was drawn during the continuation of anoxia (respiration was generally considerably depressed by this time). Due to the poor condition of the anoxic rats, portal and hepatic vein blood were not obtainable in every case. In another group, the common bile duct was ligated close to the liver, well above the entrance of the pancreatic ducts. These rats were sacrificed at intervals of 24 to 96 hours.

Results. Table I demonstrates that in the normal, fed rat, the portal vein plasma DNase activity is considerably higher than that of the hepatic vein and of the aorta. Circulation through the liver results in the extraction of 73 ± 4% of the portal vein plasma DNase.

In the normal rats, the bile demonstrated DNase activity similar to or higher than the portal vein plasma. For the 5 cases where both were examined, statistical test indicates that the portal vein and bile DNase do not differ significantly from each other.

Rats given CCl_4 24 hours before operation appeared ill. The livers were mottled and diffusely pitted. Ecchymoses occurred readily on handling. The pancreas appeared grossly

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TABLE I. DNase Activities in 16 Normal Rats.

	DNase activities in units × 10^{-4}/ml					
	Portal vein	Hepatic vein	% extraction*	Aorta	% extraction*	Bile
	600	30	95			
	296	48	84	61	79	
	233	76	67			
	222	8	96	30	86	
	209	38	82			
	198	15	92			
	171	61	64			
	† 114	27	76	49	57	
	114	42	63			
	108	53	51			
	105	38	64			
	104	30	71	57	45	144
	89	53	40			62
	76	8	89			159
	‡ 69	33	52			66
	59	15	75			96
Mean	173 ± 35	36 ± 5	73 ± 4	49 ± 7	67 ± 9	105 ± 20

* Compared to portal vein.
† Rat wt 500 g.
‡ " " 535 g.

TABLE II. DNase Activities in Rats Injected with Carbon Tetrachloride.

	DNase activities × 10^{-4} units/ml			
	Portal vein	Hepatic vein	% extraction	Bile
24 hr				
	472	453	4	
	418	433	–4	
	342	318	7	19
	129	122	5	
	* —	152	—	
	130	133	–2	48
Mean			2 ± 2	
48 hr				
	157	103	34	
	276	194	30	
Mean			32 ± 2	

* Rat wt 452 g.

atrophic. After 48 hours, the general condition of the animal appeared better, but the livers were pale, yellowish, with minute red indentations over the surface.

The rats treated with CCl_4 showed markedly diminished capacity of the liver to "extract" DNase (Table II). Twenty-four hours after injection, the portal vein and hepatic vein DNase activities did not differ significantly from each other. There appears to be slight hepatic extraction of DNase 48 hours after CCl_4 injection. In the 2 cases examined, there is 34% and 30% extraction respectively. This probably represents a significant amount of extraction, and therefore improvement over the 24 hours function ($P < 0.05$). However, the percentage extraction is still definitely below normal ($P < 0.01$). The DNase concentration in the bile of CCl_4 treated rats was markedly reduced.

Anoxia also resulted in marked reduction of the capacity of the liver to "extract" DNase (Table III). The mean hepatic vein DNase of asphyxiated rats is significantly higher than that of normal rats ($P < 0.001$) and is not significantly different from the normal portal vein DNase level ($P > 0.2$). There is, thus, no significant hepatic extraction of DNase during anoxia. Likewise, the mean aortic blood DNase of asphyxiated rats is significantly higher than normal ($P = 0.001$); and the difference between the paired portal vein and aortic plasma DNase activities is not significant in the asphyxiated rat, unlike the normal. Comparison of the percentage hepatic extraction of the paired aortic plasma DNase activities before and after asphyxia confirms the significant reduction in this hepatic function during anoxia ($P < 0.05$).

Rats with bile duct ligation showed intense icterus of foot-pads, ears, and viscera. The urine contained bile and the bile duct proximal to the ligature was distended. As noted in Table IV, hepatic "extraction" of DNase even after 96 hours obstruction remains very considerable: 68 ± 7%, which does not differ

TABLE III. Plasma DNase Activities in Asphyxia.

	DNase activities $\times 10^{-4}$ units/ml						
	Portal vein	Hepatic vein after asphyxia	% extraction	Aorta before asphyxia	% extraction	Aorta after asphyxia	% extraction
	357	—	—	99	72	190	47
	296	—	—	61	79	144	51
	226	181	20	—	—	—	—
	105	—	—	40	62	89	15
	—	111	—	—	—	—	—
	—	537	—	—	—	—	—
	—	486	—	—	—	—	—
	—	177	—	—	—	—	—
	89	93	-4	—	—	102	-15
Mean	215 ± 52	264 ± 80	8 ± 12	67 ± 17	71 ± 12	131 ± 32	25 ± 15

TABLE IV. Plasma DNase Activities in Rats Following Hepatic Duct Ligation.

Hr after ligation	DNase activities $\times 10^{-4}$ units/ml Portal vein	Hepatic vein	% extraction
24	464	106	77
72	105	47	55
96*	504	144	71
		Mean	68 ± 7

* Rat wt 430 g.

significantly from the normal mean (P >0.7).

Discussion. It is apparent from the data presented that the intact rat liver depresses DNase activity in the plasma which is brought to it by the portal vein. This is a hitherto unrecognized hepatic function. In part this extraction appears to be accomplished by excretion of DNase in the bile, since bile contains DNase at a fairly high concentration. However, other mechanisms may also be active. Indeed, biliary excretion is very likely not the principal cause for the reduction in DNase on hepatic perfusion. Biliary obstruction for 24 to 96 hours resulted in marked icterus without loss of the capacity of the liver to "extract" DNase from the portal blood. The obvious possibilities are that the liver contributes an inhibitor of DNase(5) to the blood, that it inactivates the enzyme by destroying it, and that it sequestrates it (possibly for its own metabolic use). Experiments which appear to exclude the first possibility, but make the last plausible because of the very high content of DNase active at pH 7.5 in liver homogenates, amounting to several times the concentration in serum (contrary to the results of Webb(6)), will be reported later.

Hepatic damage, due to carbon tetrachloride or anoxia, results in the loss of the capacity to "extract" DNase from the portal blood. This may be compared to the effect of hepatitis in human patients. In the latter we have observed an increase in peripheral blood serum DNase. The results presented here suggest that this is due to failure of the diseased liver to "extract" the DNase brought to it in the portal circulation.

The source of the serum DNase is not demonstrated by these studies. It may be pancreatic in origin, since it has the same pH optimum as pancreatic DNase(7). The absorption into the portal circulation may be directly from the pancreas or from the pancreatic secretion in the bowel. "Recirculation" from the bile to the portal veins draining the bowel may also contribute. Other sites of serum DNase production are not excluded. Studies are in progress to determine this.

A more detailed report, including histological observations, effect of diet, prolonged biliary obstruction and stimulation and suppression of pancreatic secretion will be presented later.

Summary. 1. DNase activity was determined by the methyl green method at pH 7.5 in the portal vein, hepatic vein and aortic plasma and bile of fed, normal (except for the anesthesia and terminal surgery) albino rats, and of rats rendered anoxic, injected with carbon tetrachloride, or made icteric by hepatic duct ligation. 2. The hepatic vein plasma regularly demonstrated lower DNase activity than did that of the portal vein in normal rats (73% hepatic extraction). 3. In normal rats, the

bile demonstrated DNase activity similar to that of the portal vein plasma. 4. Hepatic damage by anoxia or carbon tetrachloride injection suppressed the capacity of the liver to "extract" DNase from the blood. This hepatic function is abolished 24 hours after the injection of CCl_4; after 48 hours there appears to be partial recovery. 5. Hepatic duct obstruction for as long as 96 hours did not impair the liver's capacity to "extract" DNase. Thus, excretion of DNase in the bile is probably not the principal means by which it is "cleared" from the portal blood.

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Modification of Thyroid Activity in Absence of the Anterior Pituitary Gland.* (20732)

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It is generally stated that the principal, if not the only, regulatory factor governing the metabolic activity of the thyroid gland is hypophyseal thyrotrophic hormone (TSH). However, it is well known that thyroid function does not altogether cease after ablation of the hypophysis, for example, Morton *et al.*(1) noted that although the thyroids of hypophysectomized rats concentrated far less radioiodine than did those of intact animals, conversion of iodide to diiodotyrosine readily occurred. Synthesis of thyroxine did, in fact, proceed in the hypophysectomized rat, albeit markedly decreased.

Factors influencing this apparent "intrinsic activity" are, however, unclear. It has been reported that a diet low in iodine content exerts a stimulatory effect on the thyroid gland of the hypophysectomized rat. Chapman(2) stated that increased thyroid gland weight, acinar cell height, and vascularity occurred in hypophysectomized rats placed on an iodine-deficient diet. Because of the importance of this observation, relative to the elucidation of factors other than pituitary TSH affecting thyroid activity, it was deemed of interest to reinvestigate this problem, utilizing additional methods of study.

Experimental. Six-week-old male Sprague-Dawley rats, raised on Purina Lab Chow, were hypophysectomized by the standard parapharyngeal approach and maintained post-operatively on non-raised cages (following I^{131} injection, rats were placed on raised cages to prevent access to urine and feces). Five per cent sucrose in the drinking water was supplied *ad lib.* One week post-operative the rats were placed on Remington(3) diet No. 342 (containing 2% desiccated liver) which was (with the ingredients used here) found to contain 0.1 γ iodine/g by chemical analysis. Addition of iodine to the diet was carried out by preparation of an aqueous solution of NaCl and NaI; this was evaporated to dryness, pulverized to a fine powder with mortar and pestle, added to the basic diet, and distributed with a mechanical mixer. Final iodine content of the diet was further checked by analyses of random samples. In Exp. I, hypophysectomized rats

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TABLE I. Influence of Dietary Iodine on Thyroid Gland of the Hypophysectomized Rat.

			Thyroid gland			
Exp.	Iodine content of diet,* γ/g	No. of rats/group§	Wt, mg/100 g body wt	I^{131} content,‡ cts/mg/sec.	I^{127} content, mg/100 g	Acinar cell ht
I	2.5	9 (15)	5.2 ± .4†	22.4 ± 3.2†	—	3.68 ± .16
	.1	9 (15)	4.7 ± .4	126 ± 34	—	4.28 ± .43‖
II	1.0	9 (15)	6.0 ± .6	43.0 ± 12	57 ± 9†	—
	.1	9 (15)	6.2 ± .5	132 ± 75	59 ± 10	—

* On diets for 4 wk following 1 wk on Purina Lab Chow post-operatively.
† Mean ± S.D.
‡ Exp. I: Carrier free I^{131}.
Exp. II: $I^{131} + 5.0\ \gamma\ I^{127}$ carrier.
§ Figures in parentheses represent original No. of rats at start of exp.
‖ $P < .05 > .01$.

TABLE II. Influence of Dietary Iodine Content on Thyroid Gland of Intact Rats. 6 rats in each series.

	Thyroid gland	
Iodine content of diet, γ/g	Wt, mg/100 g body wt	I^{131},† % of inj. dose
1.0	7.0 ± .2*	15.0 ± 3.7*
.1	10.1 ± .2	40.5 ± 4.3

* Mean ± S.D.
† 24 hr after inj.

were placed on the low iodine diet and a similar diet augmented with 2.5 γ I^-g/m. In Exp. II, the low iodine diet was supplemented with 1.0 γ I^-/g. One month after inauguration of the special diets the rats were injected intraperitoneally with a 5 μc I^{131}. Carrier-free I^{131} was used in Exp. I. 5.0 γ I^{127} carrier was added in Exp. II. The rats were sacrificed under ether anesthesia 24 hours after injection. The thyroids were removed, and the lobes were weighed individually on a Roller-Smith torsion balance. In Exp. I, the left lobe was placed in formalin for histological examination. The other lobe was digested in 1 ml 2 N NaOH, diluted to a convenient volume and assayed for radioactivity with an end window G.M. tube. In Exp. II both lobes of the thyroid were digested in base; one aliquot was taken for I^{131} analysis and the remainder was analyzed for I^{127} content by the method described by Taurog and Chaikoff(4). The thyroids, prepared for histological study, were embedded in paraffin, sectioned at 4 μ, and stained with hematoxylin and eosin. Acinar cell heights were measured by horizontal projection of the image on a screen crossed by 3 vertical and 3 horizontal channels. All cells from a field through the center of the lobe falling within these channels were measured with a millimeter rule. Follicles cut tangentially were not included. An average of 240 cells were counted per gland. All measurements (made at x 1700) were made at one sitting by a single observer. Animals incompletely hypophysectomized were excluded from the study.

Results. It will be seen from Table I that in both experiments, maintenance of hypophysectomized rats on a diet low in iodine resulted in a significant increase in the I^{131} content of the thyroid gland 24 hours after injection of either carrier-free I^{131} (Exp. I) or I^{131} with 5.0 γ I^{127} carrier (Exp. II). No increase in the weight of the thyroid glands was observed in the groups on the iodine deficient diet (Table I). As can be seen from Table II, intact rats placed on the iodine deficient diets develop thyroid glands 60% larger than those of intact rats on the same diet augmented with 1.0 γ I^-/g. It will also be seen that a 3-fold increase in thyroid I^{131} content occurs in the former situation.

Gross examination of the thyroids failed to reveal any significant differences between the various groups of hypophysectomized rats in respect to vascularity, color, or consistency. By cytological examination, the thyroid glands from the hypophysectomized rats maintained on the low iodine diet were not distinguishable from those removed from rats given the iodine supplement, judging from vascularity, colloid content, and gross estimate of acinar cell heights. However, as seen in Table I, actual measurement suggests that the height of the acinar cells was increased in the thyroids of rats maintained on the low iodine diet. The values for the acinar cell heights are given in mm (as measured at 1700x); the computed values would be 2.17 μ and 2.52 μ for the low and high iodine groups, respectively.

It should be noted that following one month on the low iodine diet, the total iodine content of the thyroid glands was not reduced (Table I).

Comment. It is evident that the maintenance of hypophysectomized rats on a low iodine diet results in an increase in thyroidal I^{131} content as compared to that of similar rats on an iodine augmented regime. This increase in thyroidal I^{131} was found 24 hours after the injection of either 5.0 γ I^{127} labeled with I^{131} or *carrier-free* I^{131}, the question of isotope dilution being obviated in the former condition. In these experiments the low iodine diet was not found to result in an increase in thyroid gland mass (in the hypophysectomized rats). Assuming that the renal clearance of iodide and the thyroidal blood flow were essentially the same in the 2 groups, one can reasonably infer that the efficiency of iodide extraction from the blood by the thyroid gland was increased(5). This apparent increase in extraction efficiency occurred in the absence of a concomitant reduction in thyroid iodine content. It was somewhat surprising to find that the total iodine content was not reduced after one month on the iodine deficient diet. As is well known, the secretion of thyroid hormone does proceed after hypophysectomy, although at a reduced rate. As pointed out recently by Money *et al.*(6), thyroid iodine content is halved in *intact* rats placed for one month on the Remington low iodine diet. It is of interest to recall here, Astwood's(7) speculation that perhaps a deficiency of iodine within the thyroid cell is, *per se*, a stimulus to it. It is possible that a relative *iodide* deficit could have existed which would not have been revealed by the determination of the *total gland iodine.*

Microscopic examination of the thyroids glands failed to reveal differences between the groups of hypophysectomized rats as judged by acinar cell height, colloid content, vascularity, follicle size, or amount of interfollicular fibrous stroma; actual measurement of the acinar cell height suggested, however, that there was a slight increase in height in the group on the low iodine diet. This change, if significant, was by no means of the order of magnitude reported by Chapman(2). Unfortunately his data do not reveal the range of values or the variation within groups.

Evidence of a different nature suggesting that the thyroid gland may be stimulated in the absence of hypophyseal TSH has also been presented by Turner and Turner(8) who noted that incubation at cold bath temperatures appeared to activate surviving thyroid slices, as judged by cytological appearance. On the other hand, *in vitro* studies in this laboratory(9) have indicated that while incubation of surviving thyroid slices at reduced bath temperature (26°C) results in no decrease in the efficiency of iodide extraction, the rates of iodinated-tyrosine and thyroxine synthesis were markedly depressed. Incubation at 41°C slightly reduced the efficiency of iodide extraction without affecting the rate of conversion of iodide to organic combination. Control values were taken at a bath temperature of 37.5°C.

Summary. Maintenance of hypophysectomized rats on a diet deficient in iodine resulted in 1) an apparent increase in the efficiency of iodine extraction from the blood by the thyroid gland; 2) no reduction in thyroid gland iodine content and, 3) a slight and questionably significant increase in thyroid acinar cell height accompanied by other morphological manifestations of increased activity. It is suggested that hypophyseal thyrotrophin is not the only factor capable of stimulating the thyroid gland.

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Effect of Pregnancy and Lactation on Granuloma Tissue Formation and Joint Permeability in Rats.* (20733)

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Hench confirmed and extended the general observation that pregnancy has a beneficial effect on rheumatoid arthritis in the human female(1). Subsequently he and his coworkers administered large doses of ACTH or cortisone to patients with rheumatoid arthritis and obtained a dramatic and prompt improvement(2). These results made it seem probable that adrenal cortical hormones of the gluco-corticoid type are the endocrine agents responsible for the improvement seen during pregnancy and stresses, although as Selye states, "the beneficial effect of pregnancy upon rheumatoid arthritis has repeatedly been confirmed, but the underlying mechanism is still obscure(3)."

In view of the complex endocrine mechanisms existing during pregnancy and their relationship to arthritis, it became of interest to make a quantitative study of the effect of pregnancy and lactation in the rat on connective tissue formation induced by the subcutaneous implantation of cotton pellets, and on the permeability of knee joint synovial membranes injured by the intra-joint injection of formaldehyde.

Materials and methods. Female rats 90-120 days of age were obtained from Sprague-Dawley, Inc., and maintained on Rockland Rat Diet. Males were placed with the females in the evening and vaginal smears were taken in the morning to detect sperm. The day on which sperm were found was considered as day 0 and the next day as day 1 after insemination. Lactation was standardized by maintaining the number of sucklings at 8 throughout the period of lactation. Cotton pellets weighing between 4-5 mg were selected from those supplied to the dental profession in package form. Each pellet was implanted through a small incision into the loose subcutaneous connective tissue according to the method reported by Meier *et al.*(4) and modified in our laboratory by Shipley and Meyer (5) who have shown that systemically or locally administered gluco-corticoids inhibit the formation of granuloma tissue around the implanted pellets. Care was taken to place the pellet in the subcutaneous pocket in such a way that connective tissues surrounded it on all sides. The skin incisions were closed with skin clips. Pellets were bilaterally implanted in both the pectoral and groin regions in one experimental series and in another experiment in the dorso-lateral neck region only. The pellets were implanted into rats on the day of insemination and on days 6, 12, 18, 24, 30, 36, and 42 after insemination. The pellets and their attendant granulation tissue (granulomas) were removed after having been in place for 120 ± 4 hours. Any one rat carried pellets during one stage of pregnancy or lactation only, and no pellet was in place more than 120 ± 4 hours. Control animals were non-pregnant female rats which were having regular estrous cycles and were implanted with pellets without regard to stage of cycle at the time of implantation. After the pellets had been in place for 5 days the rat was killed and each granuloma was dissected out of the surrounding loose connective tissue and weighed on a torsion balance. Granulomas which were

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TABLE I. Weights of Granulomas Induced by Implanted Cotton Pellets and Clearances of Para-Amino Hippurate from Injured Knee Joints of Rats during Pregnancy and Lactation.

Day pellets removed*	Avg granuloma wt ± S.E. (mg)†		Avg PAH recovery ± max dev. from mean (%)
	A	B	
Non-pregnant	65.2 ± 3.8 (6)‡	72.4 ± 3.0 (7)‡	22.2 ± 2.8(16)‡
5	74.8 ± 6.4 (6)	80.0 ± 2.0 (5)	21.6 ± 2.6 (5)
11	62.4 ± 3.5 (8)	69.0 ± 5.8 (4)	28.2 ± 3.0 (6)
17	50.0 ± 1.8 (5)	59.3 ± 2.5 (4)	41.4 ± 4.1 (6)
23	51.0 ± 2.8 (4)	50.8 ± 3.1 (5)	39.0 ± 2.2 (5)
29	63.5 ± 3.1 (4)	70.0 ± 4.5 (5)	20.8 ± 4.9 (6)
35	53.4 ± 1.7 (5)	57.4 ± 5.0 (5)	23.1 ± 2.6 (5)
41	57.0 ± 2.6 (3)	58.8 ± 4.9 (5)	19.7 ± 3.3 (5)
47	46.6 ± 2.4 (5)	63.0 ± 4.0 (2)	29.3 ± 1.1 (5)

* Day after insemination.
† Includes fluid absorbed by cotton but not cotton itself.
‡ No. of rats.
A = Pellets in pectoral and groin regions; 4 pellets/rat.
B = " " neck region; 2 pellets/rat.

obviously infected were discarded. Pregnancy was confirmed at the time of autopsy. The value for the average net granuloma weight in any group of rats was determined as follows: The initial dry weight of the cotton pellet was subtracted from the total wet weight of each granuloma removed, giving the net weight, which included the fluid absorbed by the cotton. The average net weight of the granulomas from each rat was determined, and the mean of these averages ± the standard error of the mean was calculated for each group. The method used to determine the permeability of the synovialis was developed in the rat by Aulsebrook and Meyer(6). They have shown that the intra-joint injection of minute amounts of formaldehyde decreases the clearance of sodium para-amino hippurate (PAH) which is injected into the joint cavity 18-20 hours after the administration of formaldehyde. Cortisone or cortisone acetate administered 24 hours before and 6 hours after the injection of formaldehyde prevents the decrease in the permeability and maintains the normal clearance rate of PAH.

Some of the animals carrying pellets and other comparable rats not implanted with pellets were prepared for the joint permeability tests by injecting 0.004 cc of a 1.0% solution of formaldehyde into the knee joint approximately 20 hours before the animals were killed for the removal of granulomas. The quantity of dye excreted during the 30-minute period is expressed as percentage recovery of the quantity injected into the joint cavity. The values for each group of rats are presented as the mean ± the maximum deviation from the mean observed for the group.

Results and discussion. The data for the formation of connective tissue induced by cotton pellets are tabulated in Table I, and graphically presented in Fig. 1. The data in column A, Table I, are from the experiment in which pellets were implanted in the pectoral and groin regions, 2 per region, a total of 4 per rat. It was noted during the autopsy of these rats that as pregnancy advanced the developing mammary glands extended into the regions where the pellets were implanted. This condition suggested that the decreased weight of the granulomas observed after the 11th day of pregnancy might be due to an interference in the development of connective tissue by the mammary glands. In a subsequent experiment, pellets were implanted into the dorso-lateral neck region, one on each side, 2 per rat. The values for this series are presented in column B, Table I.

A study of the values for the wet weights of the granulomas removed during pregnancy and lactation in Exp. A and B shows that granulomas removed on the 5th day of pregnancy are slightly heavier than those obtained from non-pregnant animals. After the 5th day the weight of the granulomas formed during each 5-day period decreases progressively up to about the time of parturition. The values obtained on the 23rd day are significantly lower than those obtained on the 5th day, $P \doteq .03$ (A) and .0001 (B), or from

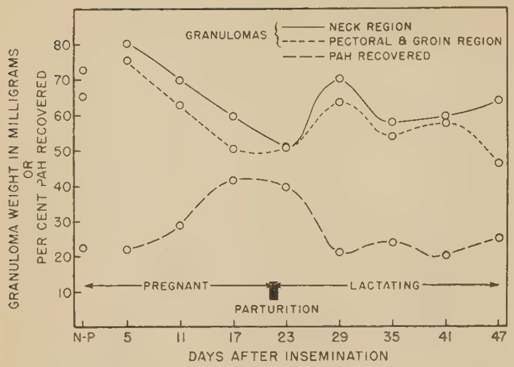

FIG. 1. Weights of granulomas induced by sub cutaneous implantation of cotton pellets and clearances of sodium para-amino hippurate from formaldehyde injured knee joints of rats during pregnancy and lactation.

non-pregnant rats, P = .03 (A) and .001 (B). Between the 23rd and 29th days post-insemination there is a significant increase in weight, P = .03 (A) and .01 (B), the values obtained on the 29th day approximating those found for non-pregnant animals. Between the 29th and 35th days post-insemination there is a decrease in values. These tend to remain low throughout the remainder of lactation. The granuloma weight decrease from the 29th to the 35th day is significant only in Exp. A where pellets were implanted in the pectoral and groin regions, P = .03 (A) and .10 (B), but the 35-day values in both Exp. A and B are significantly lower than those obtained from rats on the 5th day post-insemination, P = .02 (A) and .005 (B), and from non-pregnant rats, P = .03 (A) and .03 (B).

The weight of the relatively light granulomas obtained on the 23rd day post-insemination represents largely, we believe, fluid absorbed by the pellets very shortly after implantation, rather than tissue. In experiments which were designed to study this problem we have found that pellets of the weight used in this study soak up approximately 30 to 40 mg of fluid within 3 hours after implantation. This represents about the maximum capacity of a pellet of this weight to absorb fluid. The weight of the granulomas on the 23rd day is only 20 to 40% more than pellets holding a maximum amount of fluid, while those obtained on the 5th day weigh from 90 to 160% more than saturated pellets. The weight of the granulomas removed on the 23rd day is of the order of those found in our laboratory by Shipley and Meyer(5) for adrenalectomized rats implanted with pellets and given cortisone, hydrocortisone, 11-dehydrocorticosterone or corticosterone. Their data are similar to those reported earlier for cortisone by Meier *et al.*(4) who used cotton pellets, and by Taubenhaus and associates(7,8) who induced granulation tissues by the use of turpentine. Other investigators(9-12), utilizing other criteria, have also demonstrated that connective tissue formation is markedly inhibited by cortisone and ACTH.

It is of interest at this point to correlate the changes in the clearance of PAH from the formaldehyde injured joints of the pregnant and lactating rats with the weights of the granulomas. As the data in Table I show, the clearance values are low in non-pregnant females having regular estrous cycles and in rats on the 5th day post-insemination. After the 5th day the values progressively increase, reaching a maximum on the 17th day. Beginning just after parturition the clearance of PAH rapidly diminishes reaching early pregnancy values on the 29th day post-insemination. During the remainder of lactation the clearance values are low and show little variation from those obtained shortly after parturition.

The clearance rates observed on the 5th and 11th days are similar to those obtained by Aulsebrook and Meyer(6) in intact non-pregnant female rats in which the knee joint synovial membrane has been injured by the intra-joint injection of formaldehyde. The clearances during the period extending from late pregnancy through parturition are essentially like those found by the same authors for non-pregnant rats given cortisone just before and just after their knee joint synovial membranes had been injured by formaldehyde, or for non-pregnant rats with uninjured joints. They concluded that the injurious effect of formaldehyde, which is reflected by the impaired clearance of PAH from the joint, is prevented by cortisone.

On the basis of evidence from the literature, which is admittedly largely from studies made on the human female(13-16), it is suggested that the inhibition of granuloma formation and the prevention of the injurious effect of formaldehyde on the synovialis in the pregnant rat is due primarily to high levels of gluco-corticoids existing during pregnancy. It is further suggested that the source of the steroids is either, 1) maternal adrenals alone responding to the stress of pregnancy(17) and/or 2) maternal and fetal adrenals(18,19) (especially during later stages of pregnancy) and the placentas. The larger granulomas and the smaller clearance of PAH seen during the first week of lactation might be due to the absence of that part of the pregnancy corticoid secretion produced by the fetal adrenals and/or placentas, and to the failure of the maternal adrenals during this stage of lactation to secrete corticoids in quantities equal to those existing in late pregnancy. The smaller granulomas obtained during the remainder of lactation indicate a subsequent increase in the production of corticoids by the maternal adrenals. It is suggested, however, on the basis of the low clearance of PAH during this stage, that the level of corticoids is still less than that existing during the later part of pregnancy.

The ovary of the rat, especially the corpora lutea, may also produce anti-inflammatory substances during lactation as well as pregnancy. Preliminary data obtained in our laboratory with the cotton pellet method, however, indicate that neither progesterone, estrone, nor estradiol in amounts believed to be greater than those found during pregnancy and lactation, inhibit the formation of granulation tissue in hypophysectomized or adrenalectomized rats. Others using intact animals have obtained data which indicate that estrogens prevent the growth of connective tissue (20), and still others that they do not(21).

It may be that the ovarian steroids are part of a unique complex of interacting synergisms and inhibitions between gonadal, adrenal and placental steroids. This complex constitutes an important part of the environment in which reacting enzyme and substrate systems make for the unique biochemical pattern which characterizes the pregnant state. At the same time this complex provides a regulatory mechanism which minimizes the potential injury to the maternal cells which might result from the unopposed action of any one of the endocrine components. We have studies in progress which may yield data that contribute to our understanding of the role of steroidal complexes in regulating the mechanisms involved in resistance to stresses.

Summary. 1. Pregnancy and lactation in the rat markedly influenced the formation of granuloma tissue induced by subcutaneously implanted cotton pellets. During pregnancy there was a progressive reduction in the amount of granuloma tissue formed, the least amount being formed at about the time of parturition. After parturition there is a pronounced increase, followed by a reduction to values approximately like those obtained during late pregnancy. 2. The injurious effect of formaldehyde on the synovialis of the knee joint as measured by the clearance of sodium para-amino hippurate (PAH) is likewise progressively decreased as pregnancy proceeds, injury being almost completely absent at about the time of parturition. After parturition the PAH clearance quickly returned to the non-pregnant or early pregnancy levels. Lactation did not provide the synovialis with any measurable protection against formaldehyde. 3. The inhibition of granuloma formation and the protection of the synovialis against the injurious effects of formaldehyde are believed to be due primarily to an increase in the gluco-corticoid moiety of the unique complex of interacting steroids existing during pregnancy especially, and to a lesser extent during lactation.

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A Cutaneous Test for Rheumatic Activity in Children. (20734)

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The evaluation of activity of rheumatic fever is made clinically on the basis of Jones'(1) criteria and the combined results of such tests as antistreptolysin-O, antistreptokinase, antihyaluronidase, and C-reactive protein determinations(2-6). No single test permits estimation of rheumatic status. Our studies directed toward finding more adequate diagnostic aids led us to the work of Nassim and Banner(7), who described the absence of a cutaneous hyperemic and/or edematous response to the topical application of a 5% water-miscible cream containing the rubefacient(8-11), tetrahydrofurfuryl ester of nicotinic acid, in 5 patients with active rheumatoid arthritis. It appeared to us that the skin response to this drug might be of diagnostic value in rheumatic fever.

Materials and methods. The skin response to topical application of a vaseline-lanolin ointment containing 5% tetrahydrofurfuryl ester of nicotinic acid[*] was determined in 337 white and Negro children, several months to 18 years of age.[†] All skin tests were performed and interpreted by the authors without knowledge of the clinical diagnoses and estimates of rheumatic activity, which were made independently by the clinical or medical director of each institution. Thus the tests were done "blindly", those observing and interpreting the responses being completely unaware of the probable diagnosis, and the clinicians being unaware of the test results. Seventy mg of the nicotinic acid ester ointment was gently rubbed, by means of the examiner's finger (covered by finger cot), with 35 circular strokes into an area 1½ inches in diameter on the volar aspect of the forearm about an inch above the wrist. A control ointment was applied in the same manner, either on the forearm above the test area or

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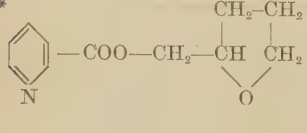
[*] 

Supplied by Ciba Pharmaceutical Products, Summit, N. J., under trade name Trafuril.

[†] These studies were carried out at our hospital, Children's Heart Hospital (Philadelphia, Pa.), Gallinger Municipal Hospital (Washington, D.C.), Irvington House (Irvington-on-Hudson, N. Y.), Jackson Memorial Hospital (Miami, Fla.), and King's County Hospital (Brooklyn, N. Y.). The authors acknowledge with thanks the co-operation of Drs. Francisco A. Hernandez, Gene H. Stollerman, Ella Roberts, Joseph Fazekas, Richard S. Gubner, Franz H. Stewart, Scheffel H. Wright and Warren W. Quillian, and the medical and nursing staffs of these institutions.

TABLE I. Diagnoses and Responses to Tetrahydrofurfuryl Ester of Nicotinic Acid of 337 Patients.

	Total cases	Typical								Atypical		
Hyperemia in ointment zone		+ or >	±				0 to —		Total	± within 10 min. fading to — in 30 min.	0 to —	Total
Edema " " "		+ or —	+		—		+	—		—	—	
Hyperemia in spread zone: >⅛" +, or >¼" ±		+ or —	+	—	+	—	+	+		—	—	
Active rheumatics	22	2	—	—	—	—	—	—	2	7	13	20
Inactive "	187*	179	3	—	1	—	1	—	184§	1	—	1
Rheumatic activity suppressed by cortisone or aminopyrine	5	5	—	—	—	—	—	—	5	—	—	—
Rheumatic activity clinically indeterminate	20	10	—	—	—	1	—	—	11	2	7	9
Normal	51†	41	2	—	2	1	—	2	48	—	2	2
Congenital heart disease	39‡	25	1	1	3	2	1	2	35	1	2	3
Misc. (a) Glomerulonephritis (subacute)	2	2	—	—	—	—	—	—	2	—	—	—
(b) Tonsillitis (including 1 case later diagnosed as scarlet fever)	3	—	—	—	—	—	—	—	—	2	1	3
(c) Others (1 case each of: hyperthyroidism, pneumonia, Riley's syndrome, psychic vomiting, bronchiectasis, pyelonephritis, skull fracture, and idiopathic endothelial fibroelastosis)	8	7	—	—	—	—	—	—	7	—	1‖	1
Total non-rheumatics	103	75	3	1	5	3	1	4	92	3	6	9
Totals	337	271	6	1	6	4	2	4	294	13	26	39

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* Including 2 cases not tabulated: one, which faded from + to — and at the same time developed edema +, and was interpreted as typical; and the other, which had a borderline reaction consisting of ± hyperemia, no edema, ± spread of ¼ inch, and was impossible to interpret.

† Including 1 untabulated case which was not readable because hyperemia was indeterminate.

‡ Including 1 untabulated case which faded from + to — and at the same time developed edema, and was interpreted as typical.

§ Includes 20 patients with elevated sedimentation rates: 11 were unexplained and 9 were attributable to other conditions, as follows: 3 diphtheria toxoid inoculation, 2 tonsillitis, 1 menstruation, 1 cystitis, 1 pyelitis and 1 dental caries.

‖ Skull fracture.

on the corresponding site of the other forearm. The *test and control ointment* areas and the *surrounding "spread zones"* were observed for hyperemia and edema, and classified as "typical" or "atypical" according to the presence or absence of the skin changes described below. In the first 75 patients, readings were made over a 3-hour period. It was found that during the first 30 minutes following inunction, the difference between the 2 types of responses was most apparent. Readings were therefore made 10, 20, and 30 minutes after application. After 30 minutes, the ointments were removed by repeated gentle wipings with alcohol pledgets, and a final reading made by comparing the test and control. If intense hyperemia developed before 30 minutes, the ointments were removed at once, and the reactions read. This rapid hyperemic response was noted particularly in infants under 2 years of age. Generally, the younger the child, the more rapid and intense was the hyperemia. No attempt was made in this study to evaluate responses in adults. Responses were best read by daylight. *Skin color changes* observed within 30 minutes were graded as: 0, blanching without edema; —, no visible color change; ±, barely perceptible hyperemia; + to ++, hyperemia of increasing intensity. The width of the spread zone reaction also was recorded. In no case was any skin reaction observed with the control ointment. Any questionable edema was disregarded. In grading the hyperemia in the spread zone, it was considered present if it was more than ¼ inch and ± in intensity, or ⅛ inch and + in intensity. Table I shows the criteria used for classifying typical and atypical responses. Generally, a *"typical"* response consisted of the development of hyperemia within 30 minutes: + to ++ in the ointment area, and — to ++ in the spread zone, extending in some cases as much as an inch beyond the site of application. Edema, with or without hyperemia, also occurred; as edema developed, hyperemia often faded, resulting in blanching. An *"atypical"* response was characterized by blanching or failure of the skin to become hyperemic at the ointment site by the end of the 30-minute period, or the development of barely perceptible (±) hyperemia within 10 minutes, fading out completely after 30 minutes. *Edema was never present in an atypical reaction.* The first 31 patients in the convalescent or active stages of rheumatic fever were followed for the effects of inunction with the nicotinic acid ester on blood count, sedimentation rate, electrocardiogram and body temperature, determined immediately before, 24 hours after, and one week following the skin test. Temperature was also recorded 3 hours after inunction. Subjective responses of the patients were noted.

Results. The types of skin response encountered in patients with and without rheumatic activity is presented in Table I. Twenty of 22 rheumatically active patients gave atypical responses, while 189 of 192 inactive patients gave typical reactions. Of the 25 patients in whom the test was performed 2 to 5 times each, the results obtained remained constant in 22 whose clinical status remained unchanged. In 3 patients whose activity status changed while under observation, the skin responses to the nicotinic acid ester reflected the changes as they occurred. One patient gave a typical response during a period of inactivity and an atypical response when she was later considered questionably active. Another child, who was originally inactive, gave a typical normal response to his first test; an atypical response to his second, when clinically active; and a typical response to his third test, when his status had reverted to inactivity. The third child presented an atypical response while active, a typical response when activity was suppressed by cortisone, and an atypical response again when the cortisone was discontinued.

No significant changes in blood count, sedimentation rate, electrocardiogram or body temperature were observed in the 31 patients in whom such determinations were made. Subjectively, patients frequently experienced mild itching and/or a feeling of warmth of short duration at the site of the test.

Discussion. The high correlation observed between the type of skin response to the tetrahydrofurfuryl ester of nicotinic acid and the presence or absence of rheumatic activity indicates that this drug may be useful in evaluating and following changes in the activity status of rheumatic patients. The present study has been limited to children, and the criteria set forth may not be applicable to adults. There are indications that readings in adults may require more than the 30 minutes we have established for final readings in children. Concentration of the ointment may be a factor.

Many more subjects will have to be studied before final evaluation can be made as to the diagnostic value of skin testing with the tetrahydrofurfuryl ester of nicotinic acid in rheumatic fever. Studies in rheumatoid arthritis and collagen vascular diseases are also indicated. The mechanism of this reaction, as well as the possible role of beta hemolytic streptococcal infection, are under investigation.

Summary. 1. Inunction with an ointment containing 5% tetrahydrofurfuryl ester of nicotinic acid (Trafuril) in 337 children caused localized cutaneous hyperemia and/or edema ("typical" reaction) in normal subjects and those with congenital heart disease or non-rheumatic conditions. 2. Twenty of 22 patients with active rheumatic disease failed to show this typical hyperemia or edema. Their response ("atypical") consisted of either blanching, failure to react, or barely perceptible hyperemia. 3. Inactive rheumatics and patients whose activity was suppressed by cortisone or aminopyrine gave typical reactions. 4. The atypical response was observed in tonsillitis and streptococcal sore throat, as well as rheumatic activity.

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Influence of Diet on Serum Alkaline Phosphatase in Rats and Men. (20735)

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The determination of serum alkaline phosphatase has proved to be valuable in the diagnosis of certain diseases of the bones and liver. There is no satisfactory explanation for the phenomena on which these tests are based. Kay(1) suggested that bone was the major source of the enzyme. Gould and Schwachman(2) came to similar conclusions from their experiments on scorbutic guinea pigs. Armstrong and Banting(3) found that removal of viscera in animals did not affect the serum phosphatase level and therefore, concluded that most of the serum phosphatase came from bone. The work of Bodansky(4) on serum phosphatase of dogs under various dietetic conditions is significant. He found that prolonged fasting greatly reduced the serum phosphatase activity of young dogs and that an increase of this enzyme was obtained only after ingestion of carbohydrates. Ingestion of cream or meat did not have any effect. Weil and Russel(5) studied the same phenomena in albino rats. They found that the plasma phosphatase in albino rats was greatly reduced by fasting. Only the ingestion of the alcohol-soluble fraction of the rat diet increased the plasma phosphatase level. The ingestion of carbohydrates and proteins did not increase the low phosphatase activity

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produced by fasting. The ingestion of saturated fatty acids and saturated dicarboxylic acids gave the same results as the ingestion of carbohydrates and proteins. An increase in the low plasma phosphatase activity produced by fasting resulted only from the ingestion of certain unsaturated fatty acids wherein a certain molecular size, a double-bond in the carbon chain, and the presence of free carboxylic groups was essential. Cephalin ingestion after fasting produced a marked increase in plasma phosphatase activity while under the same conditions lecithin was found to be entirely without action. Gould(6) also found that prolonged feeding of fats to rats resulted in an increase in serum phosphatase to extremely high levels. He suggested that the increase in serum phosphatase after fat-feeding and the decrease after fasting were due to a quantitative alteration of the enzyme rather than to the action of inhibiting or accelerating agents.

From these reports it can be seen that both in dogs as well as in rats there is marked fall of plasma phosphatase activity on fasting. While the active food factor responsible for bringing up the phosphatase activity in fasted dogs is carbohydrate, fat seems to be the important factor in rats. So far no data are available regarding the effect of diet on the serum phosphatase in men.

Methods. Albino rats were used for the first part of the experiment. They were normally fed Purina laboratory chow. The normal levels of alkaline phosphatase were estimated on samples of blood taken in the morning from animals kept on the usual diet. About 0.75 ml of blood was drawn from the tail each time and 0.25 ml of serum used in each estimation. The Binkley, Shank, and Hoagland(7) modification of the King-Armstrong method was used for the estimation of alkaline phosphatase. Twelve rats averaging 400 g in body weight were numbered and their normal serum phosphatases were estimated. They were then fasted for 24 hours and tested again for enzyme activity. Another group of 6 rats were adrenalectomized and their phosphatase levels were estimated 2 weeks after adrenalectomy both before and after fasting. Three groups of 4 rats each were selected for the feeding of carbohydrates, proteins, and fats separately. Five to 7 ml of 25% glucose solution were administered by stomach tube to one group, the same quantities of egg-albumin solution were administered to the second group, and about 5 ml of ether-extract of egg yolk were given to the third group. The serum phosphatase was estimated in each case under normal fed condition, after fasting for 24 hours, and 2 hours after feeding special diets. The same types of experiments were conducted on men. Patients from Grady Hospital who had no liver or bone diseases and who were not seriously ill were selected for the study. Three to 4 ml of blood were drawn each time and 1 ml of serum was used for each estimation. For the determination of normal levels of phosphatase, blood samples were drawn about 2 hours after the normal breakfast from patients kept on the normal diet throughout the previous day. Nine patients were tested for serum phosphatase activity under normal fed conditions. The same patients were tested again after about 20 hours of fasting. (They were not allowed to eat anything from 12 noon to 8 a.m. on the next day). Special feeding experiments were done on smaller groups of subjects. For a carbohydrate diet, a breakfast consisting of breadtoast, jam, and orange juice was allowed to 4 men who had been previously fasted for 20 hours. Blood specimens were taken before feeding and 2 hours after feeding. For a protein diet, the whites of hard-boiled eggs and skimmed milk were given. It was rather difficult to administer pure fat to patients. Hence, a diet with scrambled yolk of eggs and plenty of butter was given to the third group of subjects. Three individuals were tested with protein feeding and 6 others with fat feeding. Out of the 6 subjects fed the fat-rich breakfast, 3 were tested 2 hours after feeding, and the rest 4 hours after feeding.

Results. Table I shows that the normal alkaline phosphatase level in the younger age-group of rats is higher than that in the older ones. But in both these groups there is significant lowering of serum phosphatase on fasting for 24 hours. Adrenalectomy does not influence the effect of fasting on serum phos-

TABLE I. Serum Alkaline Phosphatase in Fed and Fasted Normal and Adrenalectomized Rats.

No. of rats	Avg wt	Serum alkaline phosphatase (King Armstrong units) Unfasted	Fasted	Difference
12	400 g (normal)	23.0±5*	11.7±2.4	11.4±2.8
6	175 g (adrex)	26.0±5.1	12.3±1.4	13.7±2.2

* Stand. dev.

TABLE II. Serum Alkaline Phosphatase in Normal Rats* under Various Conditions of Feeding.

Serum alkaline phosphatase (King Armstrong units) Unfasted	Fasted	2 hr after glucose ingestion
27.6 ± 5.6†	13.9 ± 3.3	13.2 ± 2.4
25.3 ± 4.6	12.4 ± 2.5	Y_1 12.7 ± 2.8
25.3 ± 4.6	12.4 ± 2.5	Y_2 20.1 ± 3.7

4 rats in each series.
Y_1 = 2 hr after protein ingestion.
Y_2 = " " " fat " .
* Avg wt 400 g.
† Stand. dev.

TABLE III. Serum Alkaline Phosphatase in Fed and Fasted Human Beings.

	Serum alkaline phosphatase (King Armstrong units) Unfasted	Fasted	Difference
	4.9	3.3	1.6
	4.1	1.9	2.2
	4.6	2.8	1.6
	5.2	3.0	2.2
	5.4	3.1	2.3
	8.4	4.0	4.4
	9.4	5.4	4.0
	7.5	3.7	3.8
	7.6	3.5	4.1
Avg	6.3 ± 1.9	3.5 ± 1	2.9 ± 1

phatase. Table II shows that the fall in phosphatase activity produced by fasting is not overcome by feeding either carbohydrates or proteins, whereas on feeding of fat the phosphatase activity returns to very near the normal level. Table III shows that the normal serum alkaline phosphatase in humans is not as high as in rats. However, there is a definite lowering of the activity after fasting for 20 hours. Table IV shows that the low level of alkaline phosphatase in man due to fasting is not overcome by the ingestion of carbohydrate food or protein, but that 2 to 4 hours after feeding fat a definite increase occurs.

Discussion. Tuba and Robinson(8) have recently demonstrated that the alkaline phosphatase of rat serum is composed of a fraction which is unaffected by fasting and a second larger portion, which responds relatively quickly to fasting and to ingestion of fat. They believe that this variable or adaptive fraction is largely derived from the intestine, where it is essential in the absorption of fat. Since the alkaline phosphatase response to alteration in diet is the same in the rat and human being, it would seem likely that this determination measures the activity of several enzymes arising from different sources. Although the activity of these enzymes in the plasma is frequently associated with metabolic changes in bone(9), it is apparent that the phosphatase activity of plasma is associated with enzyme activity of other organs as well. As our understanding increases of the normal relationship of serum phosphatases to metabolic processes, the alteration of these enzymes in disease will have new meaning.

Summary. 1. Serum alkaline phosphatase in albino rats was found to be lowered on fasting and was increased by the ingestion of

TABLE IV. Serum Alkaline Phosphatase in Fasted Human Beings and on Subsequent Feeding with Carbohydrate, Protein or Fat.

	Fasted	2 hr after feeding carbohydrates
	3.1	3.1
	4.2	4.0
	4.3	3.9
	4.1	3.8
Avg	3.9 ± .35	3.7 ± .3
	Fasted	2 hr after feeding proteins
	4.0	3.7
	3.0	2.9
	3.6	3.1
Avg	3.5 ± .5	3.2 ± .6
	Fasted	2 hr after fat diet
	4.2	6.6
	3.1	5.2
	4.1	6.4
Avg	3.8 ± .8	6.1 ± .7
	Fasted	4 hr after fat diet
	2.9	5.4
	4.0	7.4
	3.3	6.2
Avg	3.4 ± .55	6.2 ± .9

fat. Carbohydrates and proteins had no effect. 2. The same phenomenon was found in human beings and factors influencing alkaline phosphatase serum levels are discussed.

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Elaboration of Steroid Hormones by Surviving Adrenal Tissue Slices Obtained from Thermally Stressed Dogs.* (20736)

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In a previous communication(1), a rapid method was described for estimating the secretory activity of surviving adrenal cortical tissue slices. Essentially, the method consisted of incubating dog adrenal cortical tissue in the animal's own serum with added antibiotics in a Warburg apparatus for 24 hours. Following incubation, the hormones were extracted from the tissue and medium, purified by florosil column and paper chromatography methods and quantitatively estimated. The amount of the individual hormones elaborated by surviving adrenal cortical tissue from normal dogs was found to be fairly constant when the value was expressed as micrograms of hormone per milligram of adrenal cortical nitrogen.

Methods. Healthy adult male dogs were anaesthetized with nembutal, shaved, and 30% of the body surface burned by multiple exposure to a bank of 43 750-watt reflector photolamps for 0.8 second(2). This exposure was sufficient to cause a third or deep second degree burn. The dogs received no supportive therapy and were allowed food and water *ad libitum*. Four dogs were sacrificed 24 hours after burning and 4 others 72 hours after burning. The adrenal glands were incubated as described(1) except that two incubation vessels were used, one with and one without corticotropin.[†] Five dogs were given large doses of corticotropin[‡] over a period of 24 hours and sacrificed immediately. Two dogs received the corticotropin intramuscularly in divided doses at 8-hour intervals, and three by continuous intravenous infusion of corticotropin dissolved in 5% glucose in water. Slices of adrenal tissue were similarly incubated.

Results. As may be seen in the Table, there was a marked increase in the amount of hormone produced by the adrenal slices obtained from dogs sacrificed 24 hours after burning compared with the values obtained by incubating adrenal slices from normal dogs. The dogs which received corticotropin for 24 hours prior to sacrifice also showed increased elaboration of steroid hormones, but the increase was less marked than in the burned animals particularly with respect to 17-hydroxycorticosterone (Compound F). There was no significant difference observed between the dogs which received the corticotropin by I.M. or I.V. injection. The adrenal slices from the dogs which were sacrificed 72 hours after burning showed

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[*] The opinions expressed herein are those of the authors and should not be construed as necessarily representing the opinion of the Naval Service.

[†] Each vessel contained 10 mg of corticotropin (Armour lot No. 128-105R, potency 1.6 times that of U.S.P. standard) supplied by Dr. Irby Bunding, Armour Laboratories, Chicago.

[‡] Approximately 7 U. S. P. units/Kg of ACTHAR®.

TABLE I. Steroid Hormones Isolated after Incubation of Dog Adrenal Tissue Slices. Figures in parentheses refer to No. of dogs per experiment.

Group	Condition of dog		Compound (μg/mg adrenal N.)				
			F	E(?)	B	A	S
A	Normal (8)	Mean	2.6	1.6	.85	.95	.76
		S.E.	.17	.10	.07	.04	.08
B	24 hr after burning (4)	Mean	4.2	2.5	1.6	1.4	1.6
		S.E.	.08	.12	.14	.06	.19
		P_A	<.001	<.001	<.001	<.001	<.001
C	72 ” ” ” (4)	Mean	1.8	1.8	.91	.85	.84
		S.E.	.03	.18	.13	.08	.22
		P_A	.01	—	—	—	—
D	ACTH treated (5)	Mean	3.4	2.3	1.6	1.4	1.3
		S.E.	.05	.22	.16	.10	.09
		P_A	<.01	<.01	<.001	<.001	<.01
		P_B	<.001	—	—	—	—

Corticotropin was added to incubating medium in these experiments. "P" values were obtained from standard formulae(9) and indicate significance of the differences from the other groups as indicated by the subscripts. They are omitted except when P<.02.

a return to control levels of hormone elaboration with the exception of the normally predominant hormone, Compound F, which was consistently lower than the untreated value.

All of the adrenal slices regardless of previous treatment responded to *in vitro* corticotropin. In our experiments, those flasks which contained corticotropin consistently showed a production of hormones about 60% greater than the controls incubated without corticotropin. We did not observe any one hormone that was particularly increased by *in vitro* corticotropin. These observations are in accord with the findings of Haynes, Savard, and Dorfman(3), and of Hofmann and Davison(4).

The identity of the compound whose rate of descent on the paper chromatograms corresponded to that of Compound E is in question because the compound recovered from the paper chromatograms failed to give the characteristic color with iodine and potassium iodide ordinarily obtained with authentic Compound E(5).§ Experiments are in progress to obtain sufficient amounts of this hormone for further analytical procedures.

Discussion. The increased production of adrenal cortical hormones after a stressing situation as evidenced by an increased excretion of 17-ketosteroid hormones in the urine has been observed by numerous investigators. In the present experiments, there was a slightly greater increase in hormone production after burning than that obtained by administering large amounts of corticotropin. This finding is of interest in the light of experiments of Selye(6) and Liddle *et al.*(7) who have presented evidence for the existence of an adrenal growth factor distinct from corticotropin. The present data suggest that stimulation of adrenocortical activity cannot be achieved by the particular dose of exogenous corticotropin employed to the same degree of intensity within a 24-hour period as a severely stressing situation.

It is of interest to note that there was no disproportionate rise in the elaboration of any particular hormone when the dog was traumatized by burning. The return of the hormone production to normal values in 72 hours is in agreement with the experiments of Weichselbaum, Margraf, and Elman(8) who found a somewhat similar condition in the 17-hydroxycorticosteroid levels of blood from patients subjected to surgical procedures. The disproportionate depression in the production of Compound F by the adrenal slices obtained 72 hours after burning in the face of normal values for the other hormones is of special interest. It is conceivable that such an alteration in the hormone pattern following severe stress may play a role in the etiology of the so-called diseases of adaptation.

Summary. Adrenal slices from dogs sacrificed 24 hours after burning showed a marked increase in the elaboration of hormones compared with the values observed for adrenal tissue from normal dogs. Prior treatment of dogs with 7 U.S.P. units of corticotropin per kg also caused the production of more hormone than untreated controls, but the increase in Compound F was significantly less than in the tissue from burned dogs. Adrenal glands from dogs which were sacrificed 72 hours after burning showed a return to control levels of hormone production with the exception of Compound F which was significantly less than the normal value.

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§ The authors are indebted to Drs. G. L. Farrell and D. H. Nelson for suggesting this possible discrepancy.

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A Simple Procedure for Separation of Prothrombin and Accelerator Globulin from Citrated Human Plasma.* (20737)

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In studies of the mechanism of blood coagulation, it is frequently desirable to make use of purified systems rather than plasma or serum from which one or more of the components have been removed. As more data become available on the various properties of the clotting components in different species, the advisability of working with systems derived from a single species become apparent. A number of practical difficulties are encountered in attempting to prepare purified fractions from human plasma by methods designed for use with bovine material. The relative lability of human accelerator globulin (Ac-G) and purified human prothrombin makes it necessary to use fresh plasma. This accentuates the difficulty of obtaining sufficient plasma to apply efficiently the methods previously used for large quantities of bovine plasma. In addition, many of the adsorption technics are applicable only to oxalated plasma, which precludes their direct use on citrated blood which is the most convenient source of large volumes of human plasma. The method described here makes it possible to prepare both purified prothrombin and partially purified plasma Ac-G from the same small (10-100 cc) samples of fresh citrated human plasma. The prothrombin is obtained in high yield and free of other known clotting factors. The procedure has the additional advantages of simplicity and rapidity; the preparation is easily completed in a single working day.

Assay methods. Prothrombin. Both the one-(1) and 2-stage(2) assays were used for measuring prothrombin. In the 2-stage method acacia was eliminated from the incubation mixture. With this modification nor-

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† We wish to acknowledge the technical assistance of Mrs. Irene M. Smyth. We wish also to express our appreciation to Dr. Fremont E. Davis, Miss Angelyn A. Konugres, and the staff of the Transfusion Laboratory, Los Angeles County Hospital, for their cooperation in obtaining the blood used in these experiments.

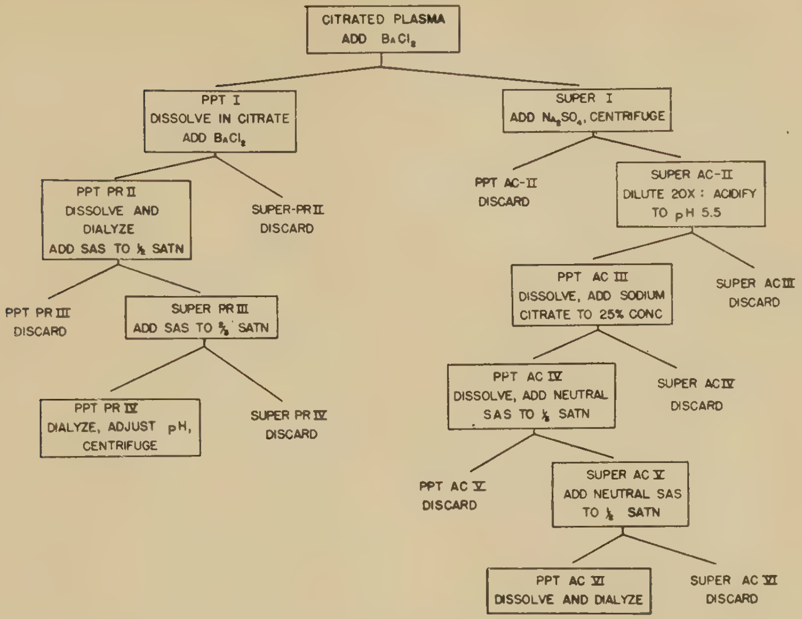

FIG. 1. Flow-sheet for preparation of prothrombin and accelerator globulin from citrated human plasma. Ppt = precipitate; Pr = prothrombin; Super = supernatant; Ac = accelerator globulin; SAS = saturated ammonium sulfate.

mal plasma was found to contain about 150 units of prothrombin per ml. *Accelerator globulin.* The one-stage assay of Lewis and Ware(3) was used for the determination of Ac-G activity.

Procedure. Prothrombin is adsorbed on a barium citrate precipitate formed by addition of barium chloride to fresh citrated plasma; accelerator globulin remains in the plasma. The barium citrate precipitate is dissolved in dilute citrate, and the prothrombin further purified by an additional adsorption, followed by ammonium sulfate fractionation. The accelerator globulin is separated from the plasma by acid precipitation, followed by precipitation in strong citrate and finally ammonium sulfate fractionation. The overall procedure is shown in the flow sheet in Fig. 1. *Preparation of prothrombin.* 100 ml cold, non-lipemic citrated plasma (citrate concentration approximately 0.02 M) are placed in a centrifuge cup of 125 ml capacity. Ten ml of 1 M barium chloride are added dropwise, with shaking, and the mixture inverted once or twice to mix. The tube is allowed to stand in the cold for 10-15 minutes and then centrifuged in a refrigerated centrifuge at 3000 g for 25-30 minutes. The supernatant fluid is decanted and saved for the isolation of Ac-G. The precipitate is dissolved at room temperature in sufficient 0.02 M sodium citrate in saline to give a volume of 100 ml. A small amount of insoluble residue remains, which is removed by centrifugation for a few minutes at low speed. The dissolved Precipitate I (Fig. 1) is decanted into another centrifuge cup, and 10 ml of 1 M barium chloride are added dropwise. After standing in the cold for 10-15 minutes, the tube is centrifuged for 25-30 minutes at top speed. The supernatant is discarded, and the precipitate (Pr-II) is suspended in sufficient 0.02 M sodium citrate in saline at room temperature to give a volume of 50 ml. The suspension is immediately divided into 2 portions of 25 ml each, and each portion is transferred to a dialysis tube about 24 inches in length and having a folded diameter of at least 2 inches.‡ The tubes are attached to some device which will keep the contents in motion during dialysis, and dialysis is carried out at room temperature

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‡ It is important that thin dialysis membranes be used and that the volume of suspension be kept small enough to permit efficient dialysis.

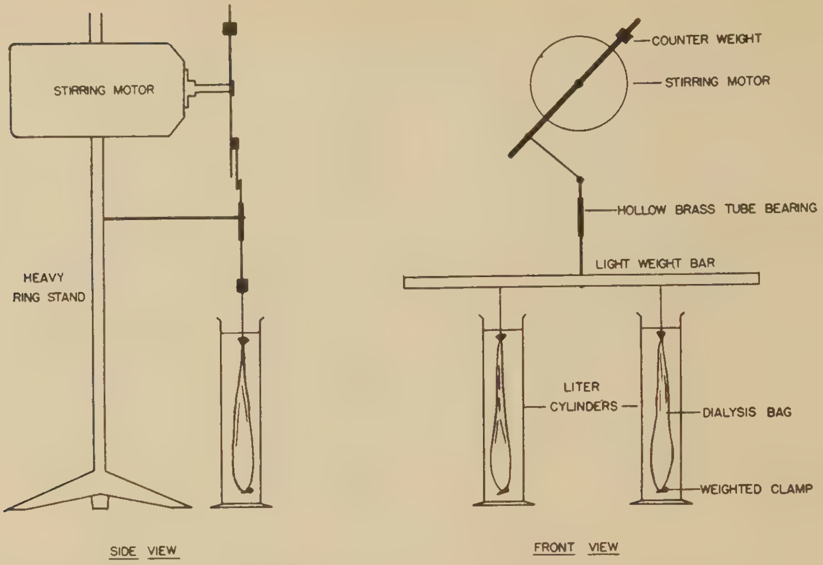

FIG. 2. Schematic drawing of device for rapid dialysis of multiple samples.

against 0.02 M sodium citrate in saline until the citrate is almost free of barium. Solution of the suspension should be complete within 25-35 minutes; an additional 1-2 hours may be required to dialyze out the barium. The dialyzed Precipitate Pr-II will be opalescent or even faintly milky in appearance. It is very important that the dialysis of this fraction be carried out at room temperature as rapidly as possible, and that the contents of the tubes be kept in constant motion during the dialysis period. Otherwise a gelatinous mass may be obtained, resulting in considerable loss of prothrombin and giving rise to difficulties in the subsequent sulfate fractionation. Good results have been obtained by using an apparatus similar to that shown in Fig. 2. The weighted dialysis tubes are suspended in one liter graduated cylinders, and the motor and bar adjusted to give a 9-inch sweep at a rate of about one per second. The dissolved, dialyzed precipitate (Pr-II) is transferred to a beaker set in an ice-salt bath, and cold saturated ammonium sulfate solution is added dropwise, with constant stirring, to a concentration of half-saturation. The mixture is centrifuged in the cold for 30 minutes at about 3000 g. The supernatant (Pr-III) is decanted into a beaker set in an ice-salt bath, and cold saturated ammonium sulfate is added dropwise, with constant stirring, to a concentration of two-thirds saturation. After centrifugation in the cold at top speed for 30 minutes, the supernatant (Pr-IV) is discarded. The precipitate (Pr-IV) is dissolved in about 5 ml of cold distilled water and dialyzed immediately against cold distilled water until free of sulfate, using some device for rapid dialysis such as described above. The dialyzed precipitate (Pr-IV) is carefully adjusted to pH 6.5 with 0.01% sodium hydroxide, and centrifuged in the cold at high speed to remove any precipitate. The supernatant fluid contains the prothrombin. The product should be clear or only very slightly opalescent.

Preparation of plasma accelerator globulin. To 10 ml of the cold Supernatant I from above, 0.75 ml of 1 M sodium sulfate is added dropwise. The barium sulfate precipitate is removed by centrifugation in the cold using high speed to secure complete sedimentation. The clear supernatant (Ac-II) is decanted into a beaker, diluted to 200 ml with distilled water, and rapidly but carefully adjusted to pH 5.5 with 1% acetic acid. The precipitate is removed by centrifugation in the cold and the supernatant (Ac-III) is discarded. The precipitate (Ac-III) is dissolved in 4.5 ml saline, and 7.5 ml of 40% sodium

TABLE I. Preparation of Prothrombin.

Fraction	Vol (cc)	One-stage assay: % of normal plasma	One-stage assay: Yield (%)	2-stage assay: Units/cc	2-stage assay: Units/mg protein*	2-stage assay: Yield (%)
Normal plasma	—	100	—	155	—	—
Starting "	800	97	—	150	2	—
Ppt Pr-I	800	120	124	195	145	130
II	400	260	133	390	860	130
III	20	160	4	460	180	8
IV	13	4000	67	6300	2210	68

* Protein measured by method of Daughaday, *et al.*(4).

citrate are added dropwise (room temperature). Centrifugation is carried out in the cold, using high speed to obtain clean separation. The supernatant (Ac-IV) is discarded. The precipitate (Ac-IV) is dissolved in sufficient chilled saline to give a volume of 2.0 ml, and transferred to a tube in an ice-salt bath; one ml of cold saturated ammonium sulfate, neutralized to pH 7 with strong ammonium hydroxide, is added dropwise with constant stirring. After centrifuging in the cold, the supernatant (Ac-V) is decanted into a tube set in a ice-salt bath, and 1.0 ml cold, neutralized saturated ammonium sulfate is added dropwise with constant stirring. Following centrifugation in the cold for 15-20 minutes at high speed, the supernatant (Ac-VI) is discarded. The precipitate (Ac-VI) is dissolved in 2 ml cold saline, and dialyzed immediately in the cold against 0.02 M sodium citrate in saline until free of sulfate.

Results. In Table I are shown the results of a large scale preparation of prothrombin which was subsequently used for electrophoresis. It will be noted that both one and 2-stage prothrombin assays indicate a yield in excess of 100% for Precipitates Pr-I and Pr-II. This phenomenon was encountered only occasionally, the usual yield at this stage of the preparation being around 80-90%. One-stage and 2-stage assays usually agree fairly well at the various stages of the procedure, but occasionally there are significant differences between the 2 assays. The final product contains a trace of thrombin but no fibrinogen or accelerator globulin by the assays used. Electrophoresis for 2 hours at pH 8.5 (sodium chloride-sodium barbital buffer, ionic strength 0.1) showed 2 components.

TABLE II. Preparation of Accelerator Globulin.

Fraction	Conc. of Ac-G (% of standard)	Yield (%)	Protein conc.(4) (g %)
Starting plasma	82	—	
Super I	74	90	
Ppt Ac-III	80	57	1.30
IV	48	29	.45
VI	37	12	.09

In Table II are shown the results of a typical preparation of plasma accelerator globulin. The preparation contained no prothrombin or fibrinogen, and could be converted to the more active serum Ac-G by thrombin. The final product represents an overall purification of approximately 35 times. As yet no preparations have been attempted which would give sufficient material for electrophoretic analysis.

Discussion. The procedure as described provides a rapid method for obtaining human prothrombin in high yield from citrated plasma. Although not electrophoretically homogeneous, the product is highly active and appears to be free of other known clotting factors. The major portion of the product prepared in this manner appears to be prothrombin. The contaminant has the properties of lipoprotein, and the concentration in the final product appears to be a function of the lipid content of the original plasma. Each milligram of protein in the products prepared in this manner from nonlipemic plasma contains the prothrombin activity of at least 10 ml of normal plasma. This is comparable to the activity of the purified human prothrombin prepared by other technics(5). It would appear, in agreement with Seegers(5), that the concentration of prothrombin in normal human plasma is somewhat lower than 10 mg %.

The preparation of accelerator globulin is obtained in much lower yield, as might be expected from the greater lability of this substance. The yield may be increased somewhat by omitting the precipitation with citrate, but the product so obtained is not so stable.

The stabilizing effect of citrate during the fractionation procedures is seen with prothrombin as well as Ac-G. The partially purified prothrombin product, Precipitate Pr-II, when dialyzed against saline and stored overnight at 4°C retains only about one-third the activity of a portion of the same preparation kept in 0.02 M citrate.

The stability of the purified prothrombin is also materially influenced by the age of the starting plasma. Preparations made from plasma which had been refrigerated or frozen for a period of several weeks have lower yields and lost one-third to one-half of their activity on overnight storage in the refrigerator. However, even when fresh plasma is used as a starting material, the stability of purified human prothrombin appears to be less than that reported for bovine prothrombin. On storage at —10°C for 3 months, solutions of human prothrombin show a steady decrease to about 20% of their original activity. Stability is not appreciably increased by drying the product from the frozen state followed by storage in the deep freeze.

Summary. A simple and rapid procedure is described for the separation of prothrombin of high specific activity and partially purified accelerator globulin from fresh citrated human plasma. Each product appears to be free from other known clotting factors.

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A One-Stage Method for the Determination of Accelerator Globulin.* (20738)

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An assay designed to measure human accelerator globulin (Ac-G) activity should meet certain criteria if dependable results are to be obtained. Of primary importance, the procedure should be simple and rapid because of the lability of human Ac-G. It should also be specific for Ac-G and unaffected by fluctuations in other clotting components in the test material. If possible, all components supplied to the test should be of human origin in order to avoid difficulty due to species differences.

Such a test system has been achieved by using as a substrate aged human plasma containing an excess of prothrombin and fibrinogen, but nearly devoid of Ac-G. Increased sensitivity to small amounts of Ac-G is obtained by using a low concentration of thromboplastin. To assure reproducible results, and to serve as a constant check on the activity of the thromboplastin, a standardized beef plasma is run with each group of tests.

Preparation of reagents. Substrate. One

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TABLE I. Progressive Deterioration of Ac-G in Aged Human Plasma during Preparation of Substrate.

Day	Clotting time (sec.) Saline	100% stand.	20% stand.
3	75	34	54
4	89	34	60
5	94	42	65
Dialyzed against 0.02 M citrate			
7	104	44	71

unit of outdated Red Cross plasma (Lilly) is reconstituted, using about two-thirds of the saline solution provided, and allowed to stand until the Ac-G deteriorates. Since deterioration takes place much more rapidly in oxalate than in citrate, the reconstituted plasma is divided into lots of 25 ml and dialyzed at room temperature against 0.02 M potassium oxalate in saline for a period of 2 to 3 days. The contents of the dialysis bags are then pooled, placed in the refrigerator at 4°C, and checked daily for prothrombin and Ac-G.‡ Prothrombin is measured by the one-stage technic of Ware and Stragnell(1). If, as occasionally happens, the prothrombin drops below 60% of normal, the material is discarded. To follow the drop in Ac-G activity, a small aliquot of the plasma (5-10 ml) is adjusted to pH 7.4 with 0.01 N hydrochloric acid, and then tested by using as a substrate in the system described below. The material is considered ready for use when a clotting time of 90-120 seconds is obtained when saline is substituted for the test plasma in the system. On the average, about 6 days are required for the deterioration of Ac-G (Table I). The substrate is then carefully adjusted to pH 7.4 with hydrochloric acid, and dialyzed in the cold against 0.02 M sodium citrate in physiological saline, since the presence of oxalate tends to give difficulty with the end point at slow clotting times. Portions of about 5 ml placed in tightly stoppered tubes and stored at –10°C generally prove satisfactory for a period of several months.

Thromboplastin-calcium solution. A saline-oxalate extract of acetone-dried human brain serves as the stock thromboplastin. Twenty ml of 0.9% sodium chloride containing 0.4 ml of 0.1 M sodium oxalate are warmed to 45°C. To the warm saline-oxalate solution are added 1.2 g acetone-dried human brain powder (prepared from the cerebrum after removing blood vessels). The mixture is inverted and incubated for 30 minutes at 45°C, stirring gently once during the incubation period. Avoid excessive agitation. Remove the larger particles by light centrifugation for a few minutes. The opalescent supernatant is relatively stable for a month or more when stored at —10°C. Avoid thawing and refreezing which often result in a drop in thromboplastin activity. For use in the test, one part of the stock thromboplastin is diluted with 4 parts of saline. The diluted thromboplastin is then mixed with an equal volume of 0.05 M calcium chloride and allowed to stand at 37°C for about 30 minutes before use. The best results are obtained when the clotting times with normal plasma fall between 40 and 50 seconds. It may be necessary to alter the saline dilution of the stock thromboplastin in order to maintain the clotting times in this optimal range.

Standards. Either untreated beef plasma or prothrombin-free beef plasma provides a stable source of Ac-G for use as a standard. Since the Ac-G level of beef plasmas may vary, standardization should be carried out by comparison with 5-10 normal human plasma samples in the test system described below. When kept at –10°C, the undiluted beef plasma is stable for months. It should be divided into small portions to avoid repeated freezing and thawing. Unless there is some reason to suspect deterioration, one standardization against normal human plasma is usually sufficient for a given lot of beef plasma.

Preparation of sample. Plasma. Blood is collected by a clean venipuncture. Nine volumes of blood are mixed immediately with one volume of 3.2% sodium citrate (0.109 M). If much difficulty is experienced with the venipuncture, or if there is the slightest evidence of incipient clotting, the sample should be discarded since the formation of even small amounts of serum Ac-G will tend to give erroneous results. The plasma is separated by centrifugation as soon as possible. If the assay cannot be done immediately, the plasma should be stored in the refrigerator to avoid deterioration of Ac-G. At 4°C, Ac-G is usually stable in citrated plasma for about 6 hours. For the assay, one volume of the plasma is diluted with nineteen volumes of 0.9% sodium chloride. *Plasma fractions.* If materials other than plasma are to be assayed, suitable dilutions in saline are prepared so that the clotting times in the assay will fall within the range covered by the standard curve. Interference by large amounts of barium, citrate, calcium, or other similar contaminating ions should be avoided by preliminary dialysis of the sample against 0.02 M sodium citrate. The dialyzed sample should then be diluted 1:20 with saline before introducing it into the assay system. *Assay procedure.* The test is carried out in a water bath or a constant temperature block kept at 37°C. The substrate and thromboplastin-calcium mixture are warmed to 37°C before beginning a series of assays, and may be kept at this temperature for several hours for convenience in running a large number of tests. More consistent results are obtained if the diluted plasma sample is also warmed to 37°C just before adding it to the reaction mixture. *Running the test.* 1) In a clean 10 x 75 mm glass tube, place 0.1 ml substrate. 2) Add 0.1 ml plasma (diluted 1:20 with saline). 3) Blow in 0.1 ml thromboplastin-calcium mixture, mix, and note the time for clot formation after addition of this reagent. 4) Determine the Ac-G content of the sample by reference to a standard curve.

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‡ As an alternate procedure, add 9 vol. of fresh blood to 1 vol. of 0.1 M oxalate. Separate the plasma and age at 4°C in an open container for 2 to 4 weeks.

Preparation of standard curve. 1) Determine the clotting times of 5-10 fresh, normal human plasma samples (diluted 1:20 with saline) in the assay system described above. The average of these values represents the clotting time of the 100% standard. 2) Make a 1:100 dilution in saline of the same normal plasma samples, and determine the clotting times. Average the values to obtain the clotting time of the 20% standard. 3) Determine the dilutions of the stock beef plasma which will give the same clotting times. This beef plasma may then be used as a reference standard. 4) Plot the results on logarithmic paper, with per cent standard as the horizontal axis and plotting time in seconds as the vertical axis (Fig. 1). Since the values between 10 and 100% fall on a straight line when plotted on logarithmic paper, the use of only two points to construct the curve is adequate. For Ac-G values which lie appreciably above the 100% level, a greater dilution of the test material should be employed in order to bring the clotting times within the range covered by the standard curve.

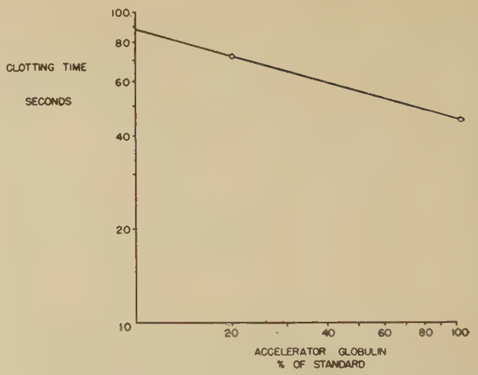

FIG. 1. Standard curve plotted on logarithmic paper.

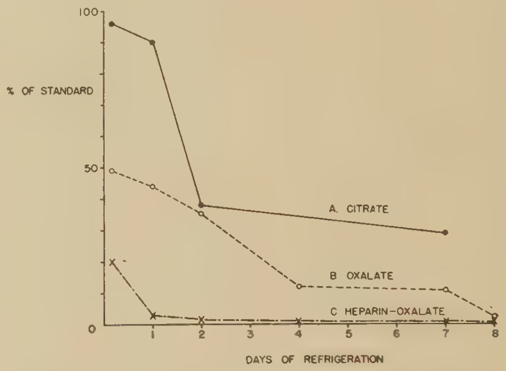

FIG. 2. Relative stability of human plasma Ac-globulin in various anticoagulants. 9 vol freshly drawn blood added to 1 vol of each of following: A. 3.2% sodium citrate. B. 0.1 M potassium oxalate. C. 0.1 M potassium oxalate containing 100 μg heparin/ml.

Stability in various anti-coagulants. The stability of human plasma Ac-G is markedly affected by the type of anticoagulatnt used. As shown in Fig. 2, stability is greatest in citrate, while appreciable deterioration takes place in a matter of hours in oxalate. The presence of the small amount of heparin recommended in the anticoagulant for the modified one-stage prothrombin test(1) hastens deterioration. After 24 hours in this mixture, the activity had already dropped too low for accurate quantitative assay, and in a few days the clotting time in the Ac-G assay system was approximately the same as the saline control. Rapid deterioration of Ac-G also takes place when versene is used as the anticoagulant. This finding is in agreement with the report of Olwin(2).

Discussion. The substrate provides an excess of both prothrombin and fibrinogen so that the test is not influenced by reasonable fluctuations in the level of these substances in the material to be tested. Dependable results are obtained on plasma from which the prothrombin has been removed by adsorption, as well as with prothrombin concentrates containing many times the prothrombin content of normal plasma.

In the actual performance of the test, failure to allow a reasonably constant interval of incubation in the constant temperature bath or block before picking up the tube to observe the end point may give rise to erratic results.

When beef plasma is used as the standard, the clotting time of the 20% standard should be determined within a few minutes of making the dilution, since an appreciable drop in activity may occur. This is particularly true of the more dilute standard, although an occasional lot of beef plasma has been encountered which seems to be labile even at a dilution employed for the 100% standard. The cause for this occasional instability has not been determined.

Summary. A simple one-stage procedure has been devised for the determination of accelerator globulin activity in human plasma. The components of the assay system are of human origin. The test is specific for accelerator globulin activity, and is unaffected by large fluctuations in prothrombin or fibrinogen content of the material to be tested.

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Action of Shellfish Poison on Peripheral Nerve and Skeletal Muscle. (20739)

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(Introduced by J. B. Bateman.)
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Shellfish poisoning, the paralytic type of poisoning which follows the ingestion of shellfish which have ingested poisonous plankton, has been adequately described by Sommer and Meyer(1). The causative substance of the paralysis has been characterized as a neurotoxin by Kellaway(2), who briefly described the effects of this toxic principle on peripheral nerve and muscle. He noted that this poison would render a skeletal muscle unresponsive to stimulation via its nerve just as does curare and that motor nerve endings were very susceptible to and readily blocked by this material.

The present investigation was initiated in an effort to characterize further the effects of shellfish poison on peripheral nerve and skeletal muscle.

Materials and methods. The preparations used were the sartorious muscle with its nerve and the gastrocnemius muscle-sciatic nerve of the frog, *Rana pipiens*. The apparatus and experimental protocol for recording action potentials are the same as those described previously in detail by Stover, Fingerman, and Forester(3) with the sole exception that in the present experiments the potentials were

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timed by signals obtained from a Tektronix time mark generator. These preparations, maintained at 25°C, were stimulated indirectly via the nerve with short (0.1-0.3 msec.) pulses of 1.5 volt amplitude. The electrical responses of both the nerve and the muscle could be recorded after suitable amplification from the screen of a triple beam cathode ray oscilloscope. When the muscles exhibited no response to indirect stimulation, they were stimulated directly with a 1.5 volt dry cell, and the presence or absence of even the slightest contraction was ascertained.

Experiments. The nerve-muscle preparation was removed from the frog, allowed to equilibrate for 30 minutes, and then placed in a lucite chamber in such a manner that the nerve could be stimulated and the action potentials of the nerve and muscle or muscle only could be recorded. The preparation was bathed in a frog Ringer solution of one to 5 intraperitoneal mouse units/ml of clam poison. The first noticeable effect is a gradual decrease in the height of the nerve and muscle action potentials when the nerve is stimulated. The electromyogram disappears before the nerve action potential does and there is no noticeable twitch of the muscle in response to a single indirect stimulus (Fig. 1). The first deflection in these records is the artifact resulting from the application of the stimulus. The interval of time between the short pips on the time base, bottom trace, represents one msec.

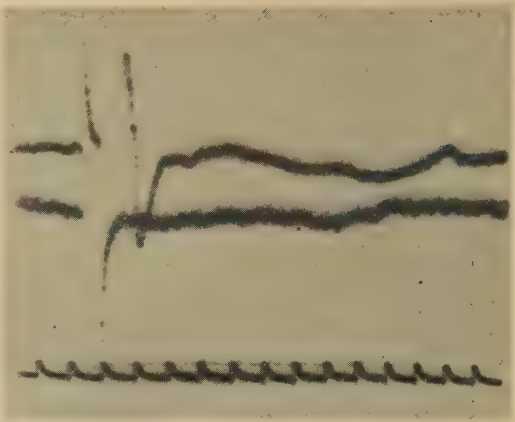

FIG. 1. A nerve action potential (upper trace) which has been recorded after the muscle has ceased to produce an action potential (middle trace). Time base (bottom trace): 1 msec. from short pip to short pip.

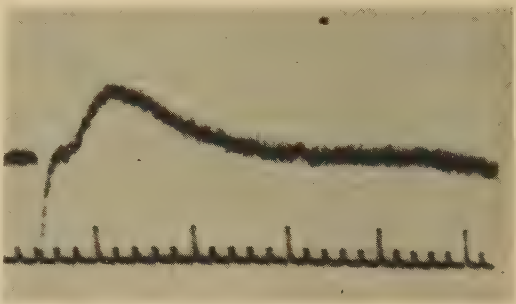

FIG. 2. An end plate potential recorded from a poisoned muscle which no longer contracts in response to a single stimulus. The electrode used to record the end plate potential is the same one used to record the middle trace of Fig. 1. Time base (bottom trace): 1 msec. from short pip to short pip.

It has been possible in 7 experiments, however, to record end plate potentials from a muscle which has just ceased to exhibit a visible contraction in response to a single stimulus (Fig. 2). If 2 stimuli are given in rapid succession, *e.g.*, at an interval of 7 msec, then the end plate potentials produced in response to each stimulus will summate to produce a muscle action potential and an associated twitch (Fig. 3). Soon these end plate potentials disappear and only the nerve action potential can be recorded. At this stage one cannot elicit a contraction in response to 2 stimuli given in rapid succession (Fig. 4). There is no evidence that shellfish poison alters the latent period of the end plate potential. The muscle was then stimulated directly with 1.5 volts and there was no response in 5 of the 7 experiments. Occasionally, however, a slight twitch was observed. Finally, the nerve also ceased to conduct an impulse.

The higher the dosage of shellfish poison, the more rapid is the sequence of events. Also the larger the muscle, the longer the time required for total paralysis. This is true because the time required to build up a lethal concentration within a large muscle is obviously greater than in a small one. For example, end plate potentials were recorded from the sartorius muscle in as short an interval of time as 2 minutes following the application of the poison. However, the gastrocnemius muscle always required a longer

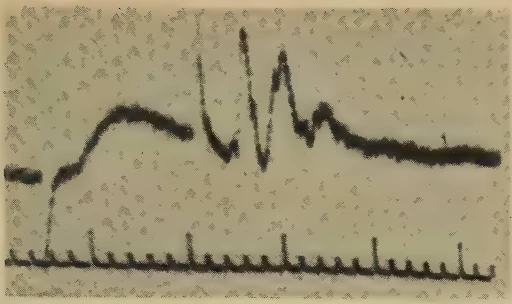

FIG. 3. The summation of end plate potentials to produce a muscle action potential. Time base (bottom trace): 1 msec. from short pip to short pip.

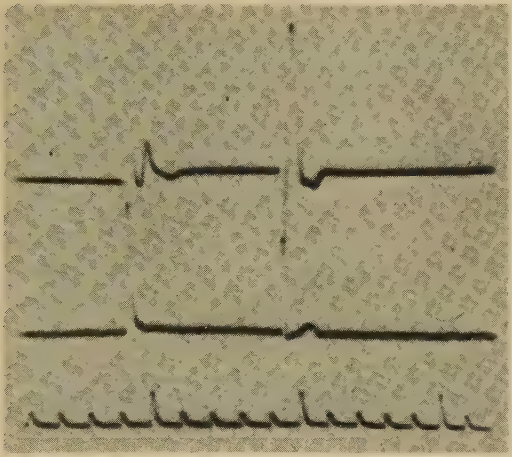

FIG. 4. After end plate potentials can no longer be recorded, the nerve action potential can still be recorded (upper trace) but no muscle action potential (middle trace) is observed even if two stimuli are applied in rapid succession. Time base (bottom trace): 1 msec. from short pip to short pip.

period. In one experiment, for example, 1.5 hours were required to block the sciatic nerve and eliminate the irritability of the gastrocnemius muscle to a stimulus of 1.5 volts.

The loss of the end plate potentials may be due to either or both of the following: (a) the terminal fibrils have been blocked or (b) the "transmitter" mechanism of the end plate has been interfered with. The former hypothesis seems more likely for Kellaway has demonstrated that the unmyelinated motor nerve endings are very susceptible to shellfish poison and are readily blocked. The relative resistance of a nerve to blocking may well be due to insulation by the myelin sheath for several investigators including Crescitelli(4) have shown that the nerve sheath is a barrier to locally applied substances. It is also obvious from these results that shellfish poison has a direct effect on muscle as well as nerve. This direct effect on nerve may well be due to diffusion in from the cut end as well as through the nerve sheath. Kellaway also observed this direct effect on muscle, but he states that the muscles did not completely lose their irritability to direct stimulation during the time they were observed.

In a second series of 3 experiments, frogs paralyzed by shellfish poison and double-pithed non-poisoned frogs were treated with 0.01 to 10 mg of acetyl choline in .1 ml frog Ringer solution. This acetyl choline solution was applied directly to the muscle or indirectly via the circulation. Their leg muscles were then observed for signs of contraction. In both instances the non-poisoned animals exhibited twitching of the leg muscles, but the poisoned animals showed no such response to the acetyl choline.

Discussion. Shellfish poison has a strong effect on both peripheral nerve and skeletal muscle. The action of this agent on muscle is similar to that of curare in 2 respects. Firstly, the poisoned muscle does not respond to stimulation via its still unpoisoned nerve and is unresponsive to directly applied acetyl choline. Secondly, end plate potentials can be recorded and summated to produce a twitch for a period of time following the application of this material. However, the effects of these dosages of shellfish poison on the isolated preparations are not reversible during an hour of observation following paralysis and the removal of the poison as are the effects of curare.

Shellfish poison has an even more general action than curare for it not only decreases the excitability of muscle to direct stimulation, but also blocks nerve conduction. In view of these actions, this agent should be referred to as a neuromuscular toxin rather than merely as a neurotoxin.

The action of shellfish poison is decidedly different from that of botulinum toxin. The latter neither blocks nerve conduction nor decreases the irritability of muscle(5) but probably inhibits the release of acetyl choline(3). Furthermore, muscle which has been paralyzed by botulinum toxin will contract in response to a dose of acetyl choline(6). The paralytic action of shellfish poison seems to be due primarily to a prevention of the muscle from responding to acetyl choline (curare-like effect), whereas the primary action of botulinum toxin is probably to prevent the release of acetyl choline.

Summary. Shellfish poison has a curare-like action, preventing the skeletal muscles from responding to liberated acetyl choline. Secondarily, it blocks peripheral nerve conduction and decreases the excitability of these muscles to direct stimulation. This material should henceforth be termed a neuromuscular toxin.

The authors' thanks are due to Dr. E. J. Schantz and Dr. J. M. Mold for placing the shellfish poison used at our disposal.

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Parallel Inhibition of Granulation Tissue by Cortisone, Hydrocortisone, Dibenamine and Banthine (R).* (20740)

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Hormonal influences upon granulation tissue formation have been well established. Stimulating and inhibiting effects can be observed, depending upon the hormone administered(1). Cortisone and hydrocortisone are preeminent as inhibiting factors and have aroused a great interest because of their clinical utility. In this laboratory it has been demonstrated that the responsiveness of small blood vessels to autonomic nervous stimuli is dependent upon the simultaneous presence of cortisone(2,3). The synergistic and potentiating effects of cortisone and certain neurohumors have been also shown to apply to a number of other phenomena, apparently not related to primary vascular mechanism. These seem to operate in the eosinophile fall(4), in certain aspects of fat transport(5) and other processes under investigation at present. Autonomic blocking agents on the other hand protect experimental animals deprived of their adrenals and subjected to stress from the ensuing shock(6). We have, therefore, investigated the effects of the adrenalins on the one hand, and of dibenamine and banthine on the other, upon granulation tissue formation in order to explore any possible relationship to the action of cortisone. To obtain comparable specimens untreated as well as cortisone and hydrocortisone treated animals were examined in the same manner, and new observations recorded.

Methods. Male, white rats of the Sprague-Dawley strain, weighing about 150 g were used. As in our previous experiments(7), one-half cc of turpentine USP was injected subcutaneously into the region of the right scapula and several animals of each series were killed daily beginning on the first to the 6th day after induction of the abscess. The dry freezing technic of tissue fixation was utilized, hoping that more definite information could be gained in regard to the structure and staining

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* Aided by a grant from the National Institutes of Health, U. S. Public Health Service.

TABLE I.

Procedure	No. of animals	Degree of inhibition*	Granulation tissue		
			Fibroblasts	Ground substance	Capillaries
Controls	12	0	Spindle shaped	Ample homogenous, containing small granules	Abundant perpendicular to abscess cavity
Cortisone, 1 mg daily	18	4+	Flat, pyknotic	Sparse, structure same	Sparse, circular to abscess cavity
Hydrocortisone, 1 mg daily	18	4+	Same	Same	Same
Epinephrine/nor-epinephrine, 1 mg daily	9	+	Same as normals	Same as normals	Same as normals
Dibenamine, 1.5 mg/100 g rat bid.	36	4+	Flat, pyknotic	Sparse	Sparse
Banthine, 10 mg/100 g rat bid.	18	4+	Same	Same	Same
Controls, starved	6	+	Same as normals	Same as normals	Same as normals
Adrenalectomized, maintained on 0.3 mg cortisone daily	6	+	Spindle shaped	Slightly reduced	Amt decreased
As above + dibenamine	6	4+	Flat, pyknotic	Sparse	Sparse
As above + banthine	6	4+	Same	Same	Same
Hypophysectomized + STH	8	+	Spindle shaped	Slightly reduced	Ample
As above + dibenamine	8	4+	Flat	Sparse	Sparse
As above + banthine	8	4+	Same	Same	Same

* 0 = no inhibition; + = slight inhibition; 4+ = strong inhibition.

characteristics of the ground substance. One portion of the abscess was fixed in formaline, another portion was immediately frozen according to the technic described by Gersh(8). The results of 17 experiments are summarized in Table I.

Results. In the untreated animals granulation tissue formation around turpentine abscesses proceeded as described before(7). The rich vascularization was again noted beginning on the second day; most of the capillaries grow perpendicularly to the abscess cavity and become abundant on the fourth and fifth day (Fig. 1A). The fibroblasts were numerous, spindle shaped, arranged parallel to the circumference of the abscess. The nuclei contained one of several distinct nucleoli and a fine meshwork of chromatin threads (Fig. 2A). In the frozen preparations the cytoplasm contained 4 to 6 or more granules strongly stained with the Hotchkiss McManus stain. These granules stained even after treatment with salivary amylase or after extraction with fat solvents. They were therefore not glycogen or fat, but considered to consist of muco polysaccharides. The ground substance also stained deeply pink with the Hotchkiss McManus stain; it was in the main homogenous but contained frequently small granules.

The effects of cortisone and hydrocortisone were reinvestigated on 36 animals half of which received a single subcutaneous injection of one mg of cortisone and the remainder one mg of hydrocortisone one day before induction and daily for five days during the development of the abscess. Both steroids had a very similar effect and the results can be discussed together. As before, a marked inhibition of the granulation tissue was observed and considerably fewer newly formed capillaries were present as compared to the response of the untreated animals(9). The direction of growth of the capillaries around the abscess cavity differed from that of the control group. On section many were seen to be cut so that the lumina were visible and fewer seem to grow perpendicularly to the abscess, indicating that the blood vessels were arranged parallel to the cavity, *i.e.* in a circular fashion (Fig. 1B). The fibroblasts differed significantly from the controls, in that they were fewer in number, smaller and containing pykotic nuclei; the

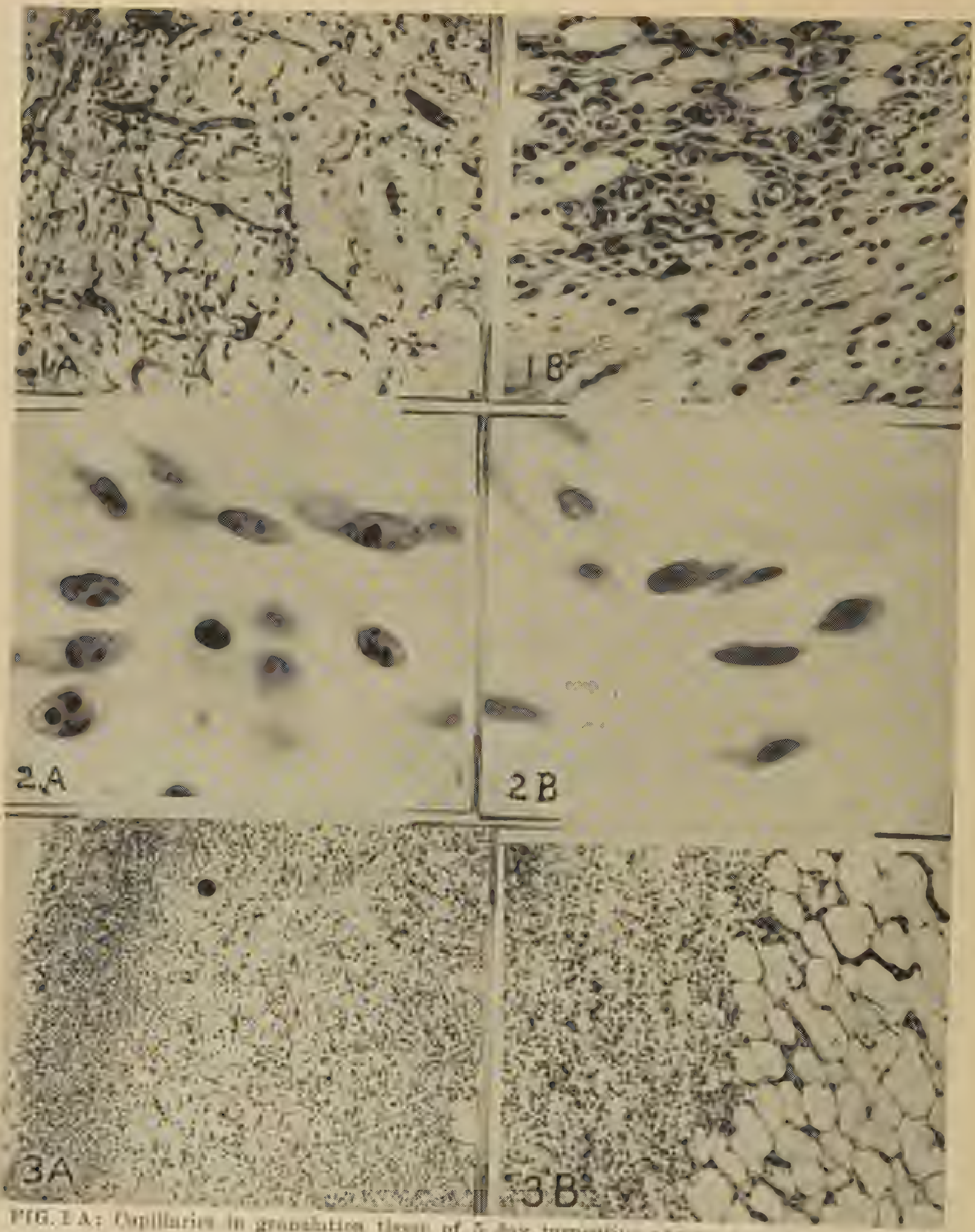

FIG. 1 A: Capillaries in granulation tissue of 5 day turpentine abscess in untreated animal (×192). Note parallel arrangement, perpendicular to abscess cavity.

B: Same in cortisone treated animal (×288). Lumina visible, capillaries arranged circularly to the abscess.

FIG. 2 A: Fibroblasts in granulation tissue of 5 day turpentine abscess in untreated animal (×1088, oil immersion). Note well defined chromatine structure and nucleoli.

B: Same in cortisone treated animal. Fibroblasts are flat and have pyknotic nuclei.

FIG. 3 A: Turpentine abscess after 5 days in control animal (×80). Leucocytic layer on the left, ample granulation tissue adjacent to it.

B: Same in dibenamine treated animal (×128). Leucocytic layer on left adjacent to fat tissue. No granulation tissue.

chromatin was arranged in denser strands, the nucleoli were less distinct and even invisible in some cells (Fig. 2B). The same number of positive Hotchkiss McManus granules were found as in the control group. The ground substance was sparser, but its finer structure and the intensity of the Hotchkiss McManus stain resembled that of the normal control group. In certain areas, however, containing few cellular elements accumulation of ground substance was noted. Some animals "escaped" the inhibiting effect of one mg of cortisone and hydrocortisone. Possibly higher doses would be necessary to produce the phenomenon with more regularity.

Commercial epinephrine in oil, containing 15-20% of nor-epinephrine, was injected subcutaneously one day before and daily after induction of the turpentine abscess into a series of nine rats. The animals were sacrificed on the 4th, 5th and 6th day. A moderate general inhibition of granulation tissue was observed. No significant changes in the appearance of the fibroblasts or of the ground substance was noted. The general arrangement of the inflammatory tissue was similar to that of the untreated animals. Cortisone and adrenaline were administered together to an equal number of animals. No modification of the inhibitory cortisone effect was noted.

Dibenamine, in amounts of 1.5 mg/100 g of rat, was administered one day before and twice daily after the injection of the turpentine. Thirty-six animals were used in these experiments and the abscesses were examined histologically every day from the first to the sixth day. No changes were observed 1 and 2 days after the induction of the abscess. The amount of necrosis, cellular infiltration and edema was about the same as in the controls. On the following days a marked to complete inhibition of granulation tissue was noted. The leukocytic layer remained unchanged but in many areas it bordered directly on the subcutaneous fat tissue, and no granulation tissue was visible (Fig. 3A and B). Newly formed capillaries were rare and in some areas absent. Fibroblasts were flat, their nuclei pyknotic, the chromatine structure was indistinct and nucleoli were not always visible. The ground substance was reduced, the staining characteristics were the same as in the normal controls. On the whole, the inhibition by dibenamine resembled that following cortisone administration but was even more uniformly obtained.

To another group of 18 animals, banthine was administered in as many injections as in the previous series. The dose was 10 mg per 100 g rat twice daily. Again a profound inhibition of granulation tissue formation was observed, even more pronounced than in the dibenamized animals, the various elements of the granulation tissue were the same as described above.

In further experiments, we attempted to explore some of the possible mechanisms of the dibenamine and banthine inhibition of granulation tissue. First it was possible that animals treated with relatively high doses of the drugs had a poor food intake and therefore, developed inferior granulation tissue. We therefore withdrew any kind of food, except water from 6 rats, while the turpentine abscess was developing. On the fourth and fifth days the absecesses were examined and showed only moderate inhibition of granulation tissue formation, not to be compared with the nearly complete one produced by the autonomic blocking agents. In another series of animals the question was investigated, whether the sympathetic blocking agents might have stimulated the adrenal cortices as non-specific stress agents. Thus, indirectly, via the action of 11 oxysteroids, the granulation tissue formation was inhibited. For this purpose, 18 adrenalectomized animals were divided into 3 equal groups; all the animals received daily doses of 0.3 mg of cortisone hypodermically, an amount which was shown to be necessary to maintain an adrenalectomized rat in good condition (10). Six of these animals were sacrificed on the 4th and 5th day after induction of the abscesses. The granulation tissue was somewhat less well developed than the normal and resembled the one previously observed in equal doses and in the same manner as detailed above. On the 4th and 5th days after injection of turpentine, granulation tissue was profoundly inhibited as seen in the intact animals similarly treated. Six other animals of this group received banthine in the dose applied before. Again strong inhibition of granulation tissue formation was observed in the absence of the adrenal cortices.

We conclude that the inhibitory influence of dibenamine and banthine is independent of the adrenal cortex and does not follow the release of corticoid hormones resulting from a stress reaction.

Another possible mechanism of action of the drugs was tested; *i.e.* whether their administration would interfere with the production and/or release of anterior pituitary somatotrophic hormone. In previous experiments (9), it was demonstrated that the latter is necessary for formation of granulation tissue. Hypophysectomy resulted in a similar lack of granulation tissue as was observed under the influence of the drugs under investigation. Therefore, the hypothesis had to be tested whether the autonomic nerve inhibitors interfere with the formation of granulation tissue by blocking somatotrophic hormone release. Such a mechanism could be postulated, because it was shown that dibenamine inhibits the release of another anterior pituitary factor, namely the luteinizing hormone(11). On the other hand, ACTH discharge is not influenced by this drug(12). Twenty-four hypophysectomized animals were maintained on daily injections of 500 mg of somatotrophic hormone.† In 8 of these animals turpentine abscesses were examined without any further medication. Two other groups (8 animals each) were given in addition banthine and dibenamine respectively. The first group exhibited fair to good granulation tissue formation indicating that somatotrophic hormone had reestablished the granulation tissue in hypophysectomized animals. Both dibenamine and banthine exerted their strong inhibitory action in the presence of the growth hormone. From these experiments, we conclude that the inhibition of granulation tissue formation by these drugs was not due to lack of somatotrophic hormone and therefore it was improbable that its release from the pituitary gland was interfered with.

Discussion. The inhibitory influence of cortisone and hydrocortisone upon granulation tissue has been reinvestigated and compared with the strikingly similar effect produced by dibenamine and banthine. The latter two drugs were chosen as representatives of sympathetic blocking agents. Diabenamine exerts its action at the ganglionic level only(13). Banthine if administered in high doses has a similar action in addition to the atropine-like effect in the periphery(14). The inhibited granulation tissue shows significant changes of the fibroblasts, and the vascular pattern. The ground substance is only quantitatively diminished. Its staining characteristics and its finer structure, if studied with the dry freezing fixation technic does not differ from the controls, indicating that the chain length of the mucopolysaccharides is not appreciably altered (15).

Some information was obtained concerning the mode of action of the drugs under discussion. They do not act by diminishing the food intake of the experimental animals. Their inhibiting effect is not a non-specific stress effect upon the adrenal cortex and it does not operate by blocking the discharge of somatotrophic hormone, which is necessary for the formation of granulation tissue. The effect then is most likely related to their pharmacologic action upon neurohumors. The observation that commercial adrenaline as administered in our experiments had no—or a slight—inhibitory influence on granulation tissue, is not in contradiction to this assumption, because of the homeostatic regulation which is operating in an intact animal after administration of this drug. As previously described granulation tissue development is only moderately altered in denervated areas(16). It must be remembered, however, that even in such regions circulating neurohumors from distant organs may very well be operating and pharmacological block of the whole animal is more effective than local denervation.

From our experiments no conclusion can be drawn in regard to a relation of the action of the sympathetic blocking agents to the hormones. Granulation tissue under conditions tested here seems to require both hormonal and neurohumoral regulation for its satisfactory development.

Summary. Dibenamine and banthine exert a strongly inhibitory influence upon the formation of granulation tissue of turpentine abscesses in white rats. It resembles the blocking effect of cortisone and hydricortisone upon such tissues. The appearance of the fibroblasts, the vascular pattern and the intercellular substance of the inhibited granulation tissue is the same, regardless of which one of the suppressing agents were administered. Some of the possible mechanisms of action of these drugs were studied.

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† Armour preparation of STH Lot No. R 527094 B was used. We are indebted to Mr. Irby Bunding of the Armour Laboratories for his generous supply of the material.

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Congenital Hypoproconvertinemia.* (20741)

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Congenital hemorrhagic diathesis associated with a prolonged one-stage "prothrombin time" (Quick test) is uncommon. Quick(1) recognized that his test was also dependent upon factor(s) other than the component usually regarded as prothrombin and substantial experimental evidence from many laboratories(2) indicates these non-prothrombin factors to be (A) proaccelerin (labile factor, factor V, AcG, etc.), (B) proconvertin (stable factor, SPCA, factor VII, co-thromboplastin, etc.), (C) fibrinogen, (D) certain inhibitors of coagulation. Additional confirmation comes from study of clinical bleeding disorders and the present communication deals with 2 new cases of congenital proconvertin (SPCA) deficiency, a condition first clearly identified by Alexander *et al.*(3) and further studied by Owren(4).

Case histories. I. A 14-year white school girl (M.T.), who had a bleeding tendency since birth, was seen in 1950. The mother had shown easy bruising and some menorrhagia. A son of one of the mother's sisters was said to be a bleeder. No defect was found in another sister and her children. No information concerning the father could be elicited. M.T. bled from the umbilical cord on the 7th day after birth and during childhood had gum bleeding, easy bruising, hematuria, and attacks of abdominal pain with pallor and jaundice. No epistaxis, hemarthrosis, hematemesis or melena were reported. Menstrual periods, starting at age 14, were frequently excessive, requiring hospitalization and transfusions. Vit. K, including synthetic K_1 (obtained through courtesy of Dr. A. Gibson, Merck & Co.) did not change the

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prothrombin time. She died in another hospital in 1952 after admission in hemorrhagic shock from which she failed to rally despite transfusions, etc. No autopsy was performed. Our physical examination (1950) revealed marked pallor, gingivitis, and ecchymoses of cutaneous area. Liver and spleen were barely palpable. Laboratory tests were carried out in 1950 and restudy of frozen (—20°C) specimens 3 years later afforded additional significant results. II. A 32-year white farmer (R.S.) with an obscure hemorrhagic diathesis was seen in 1950 and again in 1953. His father had frequent epistaxis and a distant paternal relative had "bled to death." Of the patient's 4 sons, one bruises easily, while another had frequent epistaxis. R.S.'s hemorrhagic history started with umbilical cord bleeding and easy bruising as an infant. Epistaxis was common in childhood. Since puberty he has had hematuria, melena, hematomata, and hemarthroses, but no hemoptysis or hematemesis. Vit. K, including 2 weeks on 250 mg daily of synthetic K_1 (Merck's), failed to alter his clotting defect. He has never had a blood transfusion. The residual findings are minor, chiefly impairment of right elbow and both knee joints.

TABLE I. Clotting Tests.

Procedure	M. T.	R. S.
Bleeding time	2′ 15″	1′ 15″
Tourniquet test	Negative	Negative
Platelet count	240000	240000
" "thromboplastin"	——	Normal
Coagulation time		
Glass	13′-28′	30′-88′
Silicone	45′-120′+	120′+
Clot retraction	4+	4+
" lysis	0	0
Fibrinogen, mg %	310	330
AHG assay, %	100	100
PTC " , %	90*	100
Anticoagulant titer	Negative	Negative
Antithrombin		
Plasma	Normal	Normal
Serum	"	"

* Frozen plasma tested in 1953.

TABLE II. Plasma "Prothrombin" Tests.

Test	M. T.	R. S.
Quick test, %	1-2 (73″-110″)	2-3 (48″-55″)
Proaccelerin assay, %	90	100+
Prothrombin assay		
(A) 2-stage, %	85*	103
(B) New one-stage, %	84*	108
(C) $BaSO_4$ eluate 2-stage, %	82*	105
Proconvertin		
(A) Index	3.0*	2.5
(B) Owren's assay, %	0*	0

* Frozen plasma tested in 1953.

TABLE III. Prothrombin Consumption.*

Method	M. T.	R. S.
Quick test	0	0
2-stage	79†	60
$BaSO_4$ eluate	N.D.	58

* Prothrombin consumption =

$$\frac{(\text{plasma prothrombin})-(\text{serum prothrombin})}{\text{plasma prothrombin}} \times 100$$

† Frozen serum tested in 1953.

Methods. Standard laboratory routines and tests previously described(5) need no review. *AHG and PTC assays.* Compare reductions in recalcification clotting-time (at 37°C) of 0.2 ml test standards (hemophilic or plasma-thromboplastin-component deficient plasmas, stored at –20°C) mixed with 0.05 ml patient's plasma or normal plasma dilutions. *Prothrombin* assays: include use of Soluplastin (courtesy Dr. E. W. Blanchard, Schieffelin Co.) as thromboplastin and our own tested preparations of other reagents, in methods: (A) usual AcG-modified 2-stage(6), also adding bovine proconvertin (ProC); (B) a new one-stage: 0.1 ml plasma (1:10), 0.1 ml substrate ($BaSO_4$ adsorbed beef plasma + ProC), 0.2 ml Ca-thromboplastin; (C) a new 2-stage: citrate (0.2 M) eluate of $BaSO_4$ plasma, first tested after one minute activation with optimal Ca, thromboplastin and AcG; then ProC added and activation continued to a stable clotting time. Using a standard dilution curve, unitage at one minute divided by final unitage gives "*proconvertin index*" (percentage). Owren's *proconvertin* assay (7) was also used. In the data of Tables I and II, values are expressed as percentages of the mean for a large series of normal controls.

Results. Essentially normal results (Table I) were obtained in tests of bleeding time, capillary resistance (tourniquet test), platelet count and thromboplastic function, clot re-

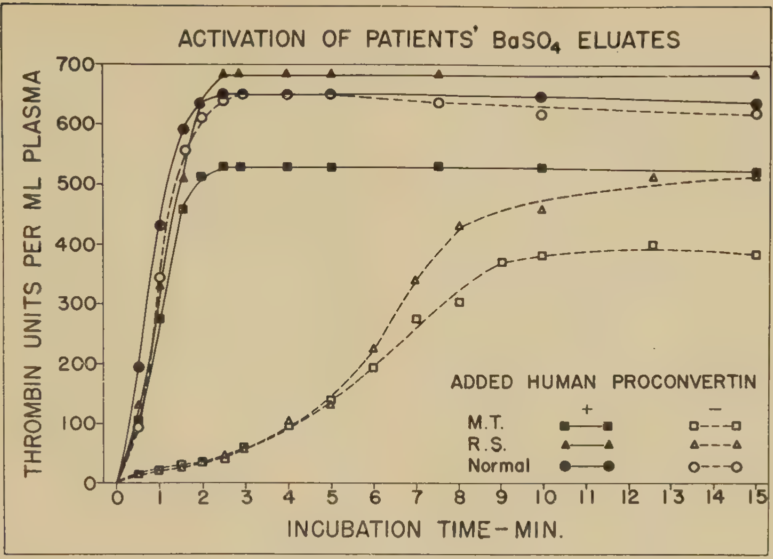

FIG. 1. Thrombin evolution from patients' $BaSO_4$ eluates in presence of optimal calcium, thromboplastin and accelerin with (+) and without (—) added human proconvertin.

traction, clot lysis, fibrinogen, AHG, PTC, and antithrombin. No circulating anticoagulant was detected. The Quick test (Table II) was extremely prolonged in both cases, corresponding to 1-3% of normal "prothrombic" activity. Proaccelerin was normal in both. The usual(6) 2-stage (A) gave prothrombin values of 63% in 6 minutes (M.T.) and 70% in 10 minutes (R.S.), compared with the normal 100 % in about 2 minutes. Activation times were shortened to normal and the prothrombin unitage significantly increased (Table II) when ProC was also added. Methods (B) and (C) gave prothrombin values which were normal and corresponded well with the proconvertin-modified (A). The proconvertin indices, 2.5-3.0, were both very low, compared with the normal 30-60. No proconvertin was detected with the Owren technic.

Prothrombin consumption tests (Table III) on the one hour (37°C) sera of M.T. and R.S. showed significant elevation of residual prothrombin. One-stage (Quick) tests could not be interpreted because plasma "prothrombin times" were prolonged also. In the 2-stage tests (C) and (A) with ProC, R.S. consumed only 60% of the initial plasma prothrombin. M.T.'s serum sample had been preserved (1950-53) at —20°C and a residual prothrombin of over 20% may be accepted as significant, since normal sera seldom contain as much as 5-10%.

As some doubt might be raised concerning the validity of the tests on M.T.'s plasma, which had been stored frozen for some 3 years, a sample of normal plasma frozen on the same date, was also tested. The concentrations of prothrombin and proconvertin were normal, but only traces of proaccelerin could be detected. This old normal plasma was employed as the control in the following experiment.

Three ml samples of oxalated plasma from the 2 patients and the "normal" were adsorbed with 300 mg $BaSO_4$; the $BaSO_4$ sedimented, washed with distilled water, and eluted with one ml 0.2 M sodium citrate. The eluates were activated with (+) and without (—) added proconvertin (human (8)) in addition to the usual calcium, thromboplastin and accelerin (Fig. 1). Without added proconvertin, thrombin formation was rapid and complete from the normal eluate, but very slow and incomplete in eluates from both patients. When proconvertin was added, little effect was noted in the normal, whereas, in the patients, both activation rates and final thrombin yields were increased essentially to normal.

Discussion. Owren's(4) nomenclature impresses us with the advantages of clarity and adaptability to differentiation of precursor (pro) and active forms. Thus, we have designated these 2 cases as hypoproconvertinemia, probably familial in type as suggested by their histories. In severity, frequency and types of bleeding episodes, these cases are indistinguishable from other kinds of congenital hemorrhagic disorders. Hence, the laboratory tests are essential for the differential diagnosis. Quick tests on both patients were markedly prolonged. Our initial examination(9) showed that this was not due to abnormality of fibrinogen, antithrombin or proaccelerin and, therefore, a diagnosis of hypoprothrombinemia, unresponsive to Vit. K, seemed justified. Re-examination of R.S. and restudy of M.T.'s frozen specimens refutes this diagnosis and establishes hypoproconvertinemia. Normal plasma prothrombin levels in both patients are clearly demonstrated by the 3 test methods, but it should be noted that the addition of proconvertin, either bovine (Table II) or human (Fig. 1) was required. The very low proconvertin index denotes a severe deficiency and its small positive value, rather than the completely negative Owren's test, is in keeping with the facts that clotting did occur, albeit slowly, in whole blood and the Quick tests. The finding of a moderate deficiency in prothrombin consumption in our cases is new. This may depend upon the severity of the proconvertin deficiency.

Summary. Two unrelated patients had a congenital, probably familial, hemorrhagic disorder associated with a prolonged "prothrombin time." Laboratory examination showed that this prolongation was not due to deficiency of prothrombin, proaccelerin, or fibrinogen, nor to excess of inhibitors. The diagnosis of hypoproconvertinemia was established by the findings of a low "proconvertin index," by Owren's test, and by the ability of added proconvertin to restore normal prothrombin conversion *in vitro*. Prothrombin consumption tests in both cases showed a moderate but significant decrease.

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Metabolic Patterns in Experimentally Induced Muscular Dystrophy.*

(20742)

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Lesions in striated and cardiac muscle have been described in animals receiving large amounts of desoxycorticosterone(1), and in animals maintained on diets deficient in vit. E, or in potassium(2). Recently Ellis noted degenerative changes in the striated muscles of rabbits treated with cortisone(3) and main-

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tained on the Rockland pellet diet which we had previously found to be inadequate in vit. E content. We have made a biochemical study of the muscular dystrophy which cortisone administration produces in the rabbit receiving adequate amounts of vit. E, and have compared these results with those observed in muscular dystrophy of vit. E-deficiency. Selye reported that many of the effects of overdosage of cortisone in rats can be prevented by simultaneous admininstration of approximately equal amounts of pituitary growth hormone(4). It appeared of interest to determine whether growth hormone would modify or prevent the dystrophy producing effects of cortisone in the rabbit.

Most of the data heretofore published on vit. E-deficiency were obtained on animals during the advanced stages of deficiency. As a result, these data are difficult to interpret since many of the observed changes could have been the non-specific result of anorexia, malnutrition and loss of muscle substance, and may give little indication of the primary metabolic changes. Therefore, in the present investigations, observations on animals during the early period following the withdrawal of tocopherol were made.

Methods and materials. Animals. Young growing chinchilla rabbits of both sexes weighing about 800 g were used. All animals were kept in individual metabolism cages during the experiments. Urinary total nitrogen, creatine, creatinine, calcium, sodium and potassium were determined daily. Urinary glucose was determined when indicated. In the cortisone experiments, each animal was observed for a control period of 7 days. Hormone injection periods lasted from 18 to 21 days. A second control period was often studied after the cessation of hormone administration. In these experiments, the amounts of urinary constituent "retained" or "lost" means the difference between the amounts excreted during a control period and during a subsequent period of treatment. In the experiments on vit. E-deficiency, the metabolic periods were separated on the basis of the level of creatine excretion. The first metabolic period was the one during which the animal was maintained on the deficient diet without obvious metabolic or clinical abnormality and lasted from 9 to 15 days. The second period was that in which creatinuria was pronounced but in which there was no change in food intake. Data from the last period preceding death, and characterized by anorexia, weight loss and paralysis, were not included.

Diets. Each day the animals were fed measured amounts of food which were constant for each animal during all periods. *Vit. E-deficient animals* were fed diet 11 of Goettsch and Pappenheimer(5) which had been treated with 1% ferric chloride. This treated diet is designated diet 13. The controls received 50 g of this diet plus 4 mg of α-tocopherol per day. This amount previously had been found to be the quantity of food which a dystrophic rabbit would eat in the early stage of the disease, and therefore, this regimen yielded a period in which the intake of the dystrophic animal was comparable to that of the non-dystrophic animal. *Cortisone-treated animals*: Group A was maintained on 70 g of the Rockland pellet diet plus 4 mg α-tocopherol per day. Groups B and C were given 60 g of diet 11(5) plus 4 mg α-tocopherol per day. These amounts of food supported growth but were slightly less than the animals would have eaten if they had been fed *ad lib* but this assured constancy of food intake throughout the experiments. Both the *Rockland pellet diet* and diet 11 were found to contain so little vit. E as to produce dystrophy and early death in young growing animals. That this was nutritional muscular dystrophy was demonstrated by the fact that the administration of vit. E reduced creatinuria and induced recovery. Histological sections of tissues of animals not receiving supplements of tocopherol revealed the characteristic lesions found in vit. E-deficiency. It will be noted in the discussion that these diets contained different amounts of sodium and potassium and different ratios of these ions. These are listed below:

	Rockland pellet diet	Diet 11
	g %	
Sodium	.06	.55
Potassium	1.52	.89
Calcium	1.04	1.14
Protein	24.0	24.8

Hormone Administration. Cortisone acetate (Merck) was injected intramuscularly into the right hind leg once daily. The dose was 10 mg per kg per day. Growth hormone (Armour) was injected intramuscularly in the left hind leg once daily. Group C-1 received 10 mg per day; Group C-2 received 20 mg per day. Since these animals all weighed about one kg, these doses were approximately equal to the amount of cortisone administered in Group C-1 and twice as large in Group C-2 respectively. *Chemical Methods.* Urinary creatine and creatinine were determined directly on undiluted urine by the semi-micro autoclave adaptation of the Folin method(6). Calcium was determined volumetrically by titration with potassium permanganate of the acid-dissolved calcium oxalate precipitate. A semi-micro Kjeldahl method was used for urinary nitrogen. Glucose excretion was estimated colorimetrically by the anthrone method(7). Sodium and potassium in the urine and in ashed samples of the diets were determined by flame photometry(8).

Results. The effect of deprivation of vit. E on the urinary excretion of creatine, creatinine, calcium, sodium and potassium is shown in Table I. The first column, showing the averages of data of seven control animals, is compared with the second column of data obtained from vit. E-deficient animals during that period after the withdrawal of tocopherol when the rabbits were growing at a normal rate and had not yet developed excessive creatinuria. The third column contains data on the same animals after creatinuria had increased and muscular performance had become impaired but food intake had not yet diminished. It may be seen that there were changes in composition of urine before the level of creatinuria was increased. The level of calcium excretion was elevated from a normal average of 23 mg per day to 70 mg, whereas the output of sodium was significantly lower than that of the control group. Although the intake of food was unchanged, the average daily rate of gain in body weight was curtailed from 25 g to 8.5 g. During the following stage, when dystrophy was well established, the amount of sodium in the urine was reduced even further; the average output at this time was only about 1/3 that of the normal rabbits. Potassium excretion increased, and although the excretion of calcium decreased, it still remained somewhat higher than that of the control animals. Other investigators have noted that the muscles of dystrophic animals contain abnormally large amounts of sodium and calcium(9,10). Our findings are consistent with these observations insofar as sodium is concerned. Our data do not account for the increased amounts of calcium found in the muscles and urine. The source of this extra calcium might be the skeleton, in view of our preliminary investigations that indicate a lower concentration of calcium in the bones of vit. E-deficient animals. The decrease in the rate of sodium excretion either coincided with, or immediately preceded, the increase in creatinuria. Fig. 1 illustrates these time relationships.

TABLE I. Effect of Deprivation of Vitamin E. Seven rabbits in each series.

		Deficient animals	
	Control animals	Before onset of dystrophy	During dystrophy
Diet (50 g per day)	13 + vit. E	13	13
Avg daily body wt gain in g	28 ± 2.4	25 ± 3.0	4.4 ± 4.5
	Avg daily urinary excretion (mg)		
Creatine	13 ± 3.1	15 ± 2.5	72 ± 4.1
Creatinine	36 ± 3.2	36 ± 3.6	38 ± 3.6
Ca	23 ± 4.9	70 ± 5.3	37 ± 6.0
Na	313 ± 12.	241 ± 19.	101 ± 14.
K	222 ± 13.	216 ± 17.	260 ± 16.

All the vit. E-deficient rabbits developed a stiffness in the extremities and back resembling that described in guinea pigs(11). Movement at the wrist and knee joints became greatly restricted. This did not seem to be due to structural changes in these joints, but rather to a decrease in the length of the muscle-tendon unit. Subsequent treatment with vit. E failed to restore full freedom of movement at these joints. The condition once produced may be irreversible or its reversibility may depend upon passive stretching or exercise as well as upon vit. E-treatment. The microscopic appearance of the skeletal muscles of these animals was characterized by coagu-

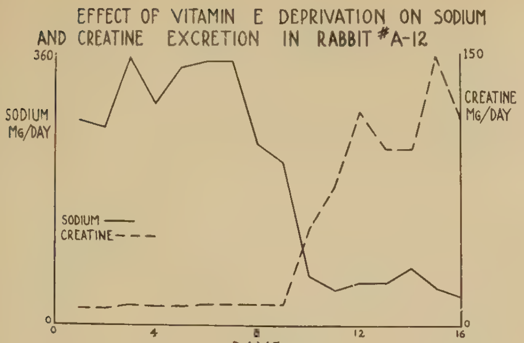

FIG. 1.

lative necrosis of the fibers, increase in nuclei of the fibers, and infiltration by adipose tissue; in short, by lesions identical to those first described by Goettsch and Pappenheimer(5).

The first two columns of Table II illustrate the effect of prolonged administration of cortisone to rabbits maintained on the Rockland pellet diet supplemented with vit. E. The daily level of urinary creatine was increased and there appeared to be an increase in urinary creatinine also. Unlike the vit. E-deficient animals, these rabbits retained little if any of the ingested sodium, and lost potassium at a much greater rate (*i.e.* about 100 mg per day). The total urinary nitrogen was increased by an average of 865 mg per day. The food intake was restricted to that of the control period; the growth rate was considerably reduced. Hyperglycemia, lipemia and glycosuria developed soon after the injections of cortisone were begun and persisted during the entire period of administration and for a time beyond the injection period.

Microscopic examination of the striated muscles of these animals showed the same types of structural change which Ellis has described(3); namely, swelling of the fibers, rupture of the sarcolemmal sheath, loss of cross-striations, and Zenker's degeneration.

The effect of identical doses of cortisone was then studied in rabbits maintained on the purified diet which, without added vit. E, induces muscular dystrophy. The animals were given adequate supplements of α-tocopherol daily, and this regimen thus permitted a comparison of the two types of muscular dystrophy, one induced by vit. E-deficiency and the other by cortisone, in 2 groups of rabbits maintained on the same basic diet. The results of these experiments appear in the third and fourth columns of Table II. The changes in the amounts of urinary constituents on this diet, due to cortisone administration, were analogous to those exhibited by animals on the Rockland pellet diet. Total nitrogen, creatine, creatinine, calcium and potassium in the urine increased and there was only a slight change in sodium metabolism. The rate of growth of the animals was very much reduced.

The muscle shortening noted in the vit. E-deficient animals did not occur in the cortisone-treated animals. Both groups of animals showed muscular weakness, and slowness in getting up after being placed on their backs. Many of the cortisone injected animals developed tremors in all 4 extremities. These

TABLE II. Effect of Prolonged Cortisone Administration on the Rabbit (10 mg/day for 3 wk).

	Group A		Group B	
	Control period	Inj. period	Control period	Inj. period
Diet	R* + vit. E	R + vit. E	11† + vit. E	11† + vit. E
No. of animals	6	4	6	3
Avg daily change body wt in g	+29 ± 3.5	—6 ± 1.7	+30 ± 2.5	+5 ± 6.5
	Avg daily urinary excretion (mg)			
Total N	685 ± 49.	1550 ± 32.	871 ± 5.8	2088 ±200.
Creatine	20 ± 1.7	67 ± 2.1	19 ± 2.5	81 ± 11.
Creatinine	35 ± 2.7	43 ± 1.7	47 ± 2.1	51 ± 3.9
Ca	22 ± 1.5	90 ± 6.0	23 ± 2.4	71 ± 22.
Na	23 ± 9.2	27 ± 3.8	294 ± 13.	280 ± 26.
K	779 ± 5.6	875 ± 16.	335 ± 8.5	455 ± 26.

* 70 g daily. † 60 g daily. R = Rockland pellets.

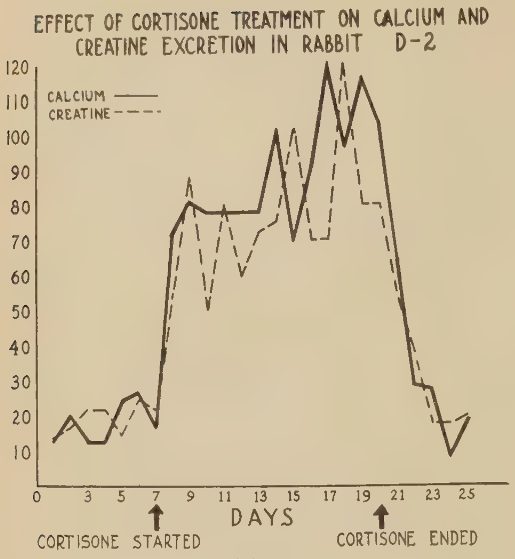

FIG. 2.

tremors were most pronounced in the group given the Rockland pellet diet. The reason for this difference is not known, but it might be the difference in K:Na ratio of the diets. In the Rockland pellet diet the K:Na ratio is 25; that of the purified diet is 1.6.

The alterations in metabolism due to cortisone administration, as reflected in urinary excretion, were prompt and were maximal by the second or third day after the first injection. Withdrawal of cortisone was followed by an equally rapid return to normal levels of urinary calcium and creatine, but the excessive excretion of nitrogen diminished gradually over a period of several days. Fig. 2 illustrates these time relationships.

It seemed possible that the pituitary growth hormone might counteract at least one of the effects of cortisone, namely, the acceleration of the rate of protein catabolism. Therefore, simultaneous treatment with both hormones might reasonably be expected to reveal the significance of this increased nitrogen loss in the genesis of the degenerative changes in striated muscle. Accordingly, cortisone-injected animals were treated with growth hormone at two different dosage levels, and biochemical studies similar to those made in the other group of animals were made in these doubly injected animals. The dose of cortisone and the food consumption remained constant. These data are presented in Table III. The simultaneous injections of growth hormone did not diminish the effect of cortisone either on creatine excretion or on histological degeneration.

When growth hormone was given in doses of 10 mg per day, the loss of nitrogen in the doubly injected animals was about 30% less than in those given cortisone alone. However, treatment with a larger amount of growth hormone (20 mg per day) not only did not enhance this partial reversal of the catabolic action of cortisone, but actually accentuated the effect of cortisone. Moreover, at this dosage level, growth hormone treatment alone, produced an increase in daily nitrogen excretion. The increased nitrogen excretion of the doubly injected animals was close to the arithmetical sum of the increases obtained with each hormone separately. Glycosuria appeared on the second day of injection of either growth hormone or cortisone and disappeared gradually after cessation of treatment. The effect of growth hormone on nitrogen excretion in vitamin E-deficient animals was also studied, and is recorded in Table IV. The food intake was included with these data because of the variability of appetite in the vit. E-deficient animals which must be taken into account in evaluating the data. The daily nitrogen excretion in vit. E-deficient animals after the onset of dystrophy was found to be the same as, or somewhat greater than, the amount excreted during the period immediately preceding other evidence of dystrophy (creatinuria and weakness). Since food consumption was decreased in all these animals, it is evident that less nitrogen was retained by the dystrophic animals. In three vitamin E-deficient animals given growth hormone, the food consumption before and during the injection periods was practically unchanged. In two of these animals, growth hormone appears to have induced a small retention of nitrogen, as well as a slight decrease in creatinuria. However, the clinical condition was not improved and the life span of either animal was not prolonged. At these lower dosage levels of growth hormone (3 to 5 mg per day), glycosuria appeared as rapidly as it did when

TABLE III.
Effect of Pituitary Growth Hormone on Urinary Nitrogen Excretion in Cortisone Dystrophy.

Group	Cortisone, mg/kg/day	Growth hormone, mg/day	Change in urinary N from control level, mg/day	Urinary glucose, mg/day
B	10	0	+1128	+ 795
	10	10	+ 841	+1680
C	0	20	+ 185	+ 810
C-1	10	0	+1217	+2420
C-2	10	20	+1363	+4150

TABLE IV. Effect of Growth Hormone on Nitrogen Retention in Nutritional Muscular Dystrophy (4 Day Periods).

	Control period			Growth hormone, 3-5 mg/day			
Animal No.	Avg daily food eaten, g	Avg daily N intake, g	Avg N excreted daily, g	Avg daily food eaten, g	Avg daily N intake, g	Avg N excreted daily, g	Difference, g
M-65	57	2.26	1.55	53	2.10	1.09	–.46
-78	34	1.35	1.00	37	1.42	1.00	0
-49	70	2.78	1.32	69	2.74	1.27	–.05

larger doses were given but was not so pronounced.

Discussion. Tocopherol deprivation and cortisone overdosage afford two different methods of studying metabolic patterns which are present in animals with progressive muscular degeneration.

Cortisone overdosage did not impair the appetite, and could be tolerated for much longer periods of time, at the dosage levels used in these investigations, than could total tocopherol deficiency. The animals with nutritional muscular dystrophy usually developed inanition in from 4 to 9 days after onset of creatinuria and all growing animals died in from 3 to 14 days after the onset of elevated creatinuria. This variation in survival time is partly related to age; as a general rule, the younger the animal, the shorter the survival time is apt to be. Older mature animals may actually develop a chronic form of nutritional muscular dystrophy and live for a considerable period. On the other hand, even young animals have survived a month of cortisone treatment, but definitive studies on the ability of rabbits to survive cortisone administration were not made.

During the period of dystrophy, the vit. E-deficient animals developed muscle shortening and paralysis, neither of which were symptoms of cortisone overdosage.

The two types of experimentally induced dystrophy were characterized by differences in the blood constituents. Cortisone treatment induced a severe lipemia that was not seen in vit. E-deficiency. On the other hand, preliminary experiments indicate that the ratios of the various blood proteins are altered in muscular dystrophy of vit. E-deprivation, with considerable increase in the third globulin component. These observations will be reported in detail later.

Disturbances in carbohydrate metabolism were observed in both forms of muscular dystrophy. Cortisone treatment produced hyperglycemia and glycosuria. In vit. E-deficiency the concentrations of glycogen in liver and muscle are decreased(12), as we have confirmed in some of our animals.

In a qualitative sense, the daily urinary excretions of creatine, nitrogen, potassium, calcium and sodium were similar in the two types of muscular dystrophy, but there were significant quantitative differences. The maximal creatinuria due to cortisone overdosage was less than that seen in vit. E-deficiency, but loss of nitrogen and potassium was greater. A significant retention of sodium was an early symptom of vit. E-deficiency, in contrast to the slight fall in sodium excretion observed in cortisone treatment. However, chemical analyses of the muscles of animals given cortisone, revealed sodium concentrations that were significantly greater than in the muscles of untreated controls(3).

Small doses of growth hormone induced nitrogen retention in cortisone treated and in vit. E-deficient rabbits. The fact that larger amounts of growth hormone have an opposite effect and result in an increased amount of urinary nitrogen, may be due to the diabetogenic action of the hormone to which the rabbit appears to have a sensitivity similar to that exhibited by the cat(13). Glycosuria developed within from 1 to 3 days days after administration of growth hormone had been started.

It has been demonstrated that cortisone administration induces an acceleration in the rate of protein catabolic processes resulting in increased nitrogen excretion and in decreased body substance(14). The question of the genesis of the muscular wasting in vit. E-deficiency still is unsettled. It has generally been supposed that the loss of muscle is due to an increased breakdown of muscle protein, but whether protein breakdown actually is increased is not known and cannot be decided by the methods employed in these studies. The possibility, however, that reduction in the rate of synthesis of normal muscle protein may be a primary defect in nutritional muscular dystrophy is suggested by our studies on the liberation of free amino nitrogen by the tissues of vit. E-deficient hamsters(15), and possibly by the alterations in the electrophoretic pattern of the blood proteins, to be reported later.

Summary. 1. A study has been made of the metabolic patterns of rabbits with muscular dystrophy due to vit. E-deficiency and to chronic overdosage with cortisone. Both conditions are characterized by an increased urinary excretion of creatine, nitrogen, potassium and calcium. These urinary losses are quantitatively greater in cortisone induced dystrophy, although the clinical symptoms of muscular weakness and diminished life span were most marked in vit. E-deficiency. 2. The effect of pituitary growth hormone administration on both types of experimental muscular dystrophy was studied. Combined with cortisone in large doses, it exacerbated the metabolic abnormalities. Smaller doses partially curtailed the increase in nitrogen excretion. Superimposed on vit. E-deficiency, growth hormone was without effect clinically and mildly active with respect to nitrogen storage for a few days. 3. Both cortisone and growth hormone were diabetogenic in the rabbit.

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Therapy of Experimental Hydrazine Poisoning. (20743)

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The increased use of hydrazine in industry has caused a renewed interest in the nature and treatment of poisoning from hydrazine. Convulsions are characteristic of early severe poisoning; with large doses of hydrazine, such as used in this study, the convulsions invariably produce death. With the prevention or amelioration of the convulsions by the use of barbiturates a depression develops beyond that due to the treatment agent, and the animals die during the depression usually with severe pulmonary edema(1). In less severe poisoning the convulsions are absent, the depression is mild and death occurs in a few days from central necrosis of the liver(2). The object in this study was to find some agent or agents which would prevent the deaths due to pulmonary edema and liver damage while using the barbiturates to control the convulsive phase. Some of the products of intermediate metabolism of glucose were selected for study as treatment agents. These were selected because Underhill had related the poisoning process to disturbances in glucose metabolism(3-5), and the brain tissue from rabbits poisoned by hydrazine gave a greater oxygen uptake with succinate than the brain tissue from normal rabbits(6).

Methods. Adult albino mice were used in all experiments. Hydrazine was given intraperitoneally. The susceptibility of this strain to hydrazine administered intraperitoneally was as follows: 50 mg/kg killed 0 of 10, 6.25 mg/kg killed 5 of 10, 75 mg/kg killed 8 of 10, and 100 mg/kg killed all of 50. The concentration of hydrazine and agents used for treatment was such that the dose of each for a 20 g mouse was contained in 0.5 ml of solution.

For screening experiments female mice were used 4 to a group, with 4 groups for each organic acid studied. Two groups received 250 mg/mouse of glucose subcutaneously before the start of the experiment, the other 2 groups received nothing. All groups then received hydrazine 195 mg/kg followed immediately by a mixture of thiopental Na 25 mg/kg and pentobarbital Na 12.5 mg/kg also given intraperitoneally. One group of the glucose treated and one group not receiving glucose received subcutaneously the sodium salt of an organic acid involved in the intermediate metabolism of glucose. The doses are indicated in Table I. The time of injection of hydrazine and time of death was noted in each mouse. The mean survival time of each treated group was divided by the mean survival time of its control group. When the quotient was 1.4 or more the experiment was repeated either with or without glucose, whichever gave the higher quotient. For the study of treatment groups of 12 mice each were used. Both males and females were used, as indicated in Table II. Hydrazine was given in doses of 100 mg/kg or 125 mg/kg. All mice received thiopental Na 50 mg/kg intraperitoneally immediately after hydrazine. Treated animals received Na pyruvate 1 g/kg subcutaneously immediately following the barbiturate, and 3 more

TABLE I. Effect of Sodium Salts of Organic Acids on Hydrazine Poisoning Treated with Barbiturates.

	Ratio of survival times, $\frac{\text{Treated}}{\text{Control}}$			
	No glucose		Glucose, 250 mg/mouse	
Citric	1.2		.4	
Glutamic	.7		1.2	
Lactic	.9		1.0	
Malic	1.5	1.0	1.4	
Fumaric	1.4	1.7	1.0	
Pyruvic	1.9	2.3	1.0	
Oxaloacetic	.9		2.0	1.5
Succinic	1.4		1.6	1.9

All mice received intraper. 195 mg/kg of hydrazine, 25 mg/kg of thiopental Na and 12.5 mg/kg of pentobarbital Na. Sodium salts of the acids were given subcut. 0.5 g/kg for Na glutamate, 1 g/kg of the acid for succinic and oxaloacetic acid, and 1 g/kg of the sodium salts for the remainder. Each group consisted of 4 mice.

TABLE II. Treatment with Thiopental Na and Na Pyruvate.

Sex	Pyruvate, hr after hydrazine	Hydrazine I.P., mg/kg	No. surviving of 12 mice	
			Controls	Treated
♀	1, 3, 5	100	0	9
♀	2, 4, 6	100	0	12
♀	2, 4, 6	125	0	8
♂	2, 4, 6	125	0	10

Thiopental Na 50 mg/kg given I.P. at time of hydrazine inj., at 1, 3, and 5 hr, 0.5 mg/mouse given to treated animals. All doses of Na pyruvate were 1 g/kg given subcut., first one immediately following hydrazine and the others as stated. Each group consisted of 12 mice.

doses at the times indicated in Table II. In addition the treated mice received thiopental Na 0.5 mg/mouse (average dose 25 mg/kg) at 1, 3, and 5 hours after the hydrazine.

Results. In hydrazine poisoned mice there was no difference in survival time between mice which received pentobarbital Na 25 mg/kg and those which received a mixture of thiopental Na 25 mg/kg and pentobarbital Na 12.5 mg/kg. However, when thiopental Na 50 mg/kg was compared with pentobarbital 25 mg/kg the average survival time for thiopental Na was 1.3 times that for pentobarbital Na. There is one chance in 20 that this difference is not significant. A potential advantage to the use of a short acting barbiturate with a poison such as hydrazine which gives a convulsant phase followed by a depressed phase also influenced us to use thiopental Na when developing the treatment, although the mixture was used in the preliminary study on survival time. Sodium citrate, glutamate, and lactate were eliminated from the study after the first 2 experiments, because average survival times of treated mice were less than 1.4 times that of the controls (Table I). After the third experiment it was seen that Na pyruvate without glucose gave the highest average survival time of approximately twice that of the controls, so pyruvate was selected for further study. It was possible by using 3 or 4 injections of the same dose of pyruvate along with added barbiturate to increase the survival time up to 6 times that of a single dose of barbiturate, but it was impossible to prevent death with any combination of pyruvate and barbiturate with the dose of hydrazine at 195 mg/kg. The use of repeated doses of pyruvate seemed to increase the need for barbiturate, but when the barbiturate was given in the same amounts to the controls the average survival time was somewhat less than with a single dose.

The final treatment was then developed using a dose of hydrazine of 100 mg/kg with thiopental Na 50 mg/kg as the anesthetic (Table II). All controls died. There were 9 of 12 survivors when pyruvate was given at one, 3, and 5 hours, at the same time as the additional thiopental Na was given, and 12 of 12 survivors when the pyruvate was given at 2, 4, and 6 hours. When the schedule of treatment was held the same as in the latter instance and the dose of hydrazine was raised to 125 mg/kg there were 8 of 12 survivors in the female mice and 10 to 12 survivors in the male. The exact mechanism of action of this treatment is not known but the severe liver damage so characteristic of hydrazine poisoning is not seen in the surviving mice and the pulmonary edema was either reduced or eliminated.

Summary. Severe hydrazine poisoning in the mouse was successfully treated by the use of thiopental Na and Na pyruvate.

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Effects on Parturition in the Rat of Ablation of Different Lobes of the Hypophysis.* (20744)

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Pencharz and Long(1,2) observed that pregnancy in the rat was not interrupted by removal of the hypophysis on or after the 11th day of pregnancy. Parturition was, however, deranged; the rats died or gave birth to dead or living young after a prolonged pregnancy. Selye, Collip, and Thompson(3) reported that gestation was prolonged to 26 days in 22 out of 24 rats hypophysectomized between 10 and 14 days of pregnancy, but that the young were born alive. Pencharz and Long(2) and Smith (4) found that the posterior lobe was not essential for gestation and normal parturition in the rat. Sufficient advances in the preparation and care of hypophysectomized animals have been made since these early studies to justify reopening the subject of the effect of hypophysectomy on the termination of pregnancy. Experience has reduced the time required for the operation and the attendant trauma to tissue. Post-operative care has been improved and includes such measures as special diet, air-conditioning and supplementary injections of glucose and antibiotics. Skill acquired in removing the anterior and posterior lobes separately with precision[†] has also made possible the reanalysis of the effects on parturition of removal of each lobe and antibiotics have been used successfully to decrease post-operative mortality.

Methods. The animals employed were female rats of the Long-Evans strain, 3 to 6 months old, weighing approximately 200 g. The parapharyngeal approach to the hypophysis was used in all operations. This report includes results from a total of 86 rats in which operative procedures were considered successful. The effects of post-operative terramycin injections were tested in 57 of these rats, which had been subjected to complete hypophysectomy on day 12 or 13 of gestation; 30 of these animals received 4 ml of 125 mg % terramycin in 5% glucose solution on the day of the operation and the subsequent day; the other 27 received no supplement after the operation. The effect on pregnancy and parturition of ablation of the posterior and intermediate lobes was studied in two ways: in 7 rats these lobes were removed 3 to 7 weeks after conception; in 10 other rats the lobes were removed on the 12th day of pregnancy. (The removal of the posterior and intermediate lobes will henceforth be designated as posterior hypophysectomy or ablation of the posterior lobe.) In order to check the effect on parturition of the absence of the anterior lobe only, this lobe was removed in 12 animals on the 12th day of pregnancy. All animals received terramycin post-operatively with the exception of those in the group of completely hypophysectomized rats which were used as untreated controls in the experiments designed to test the action of this antibiotic.

The rats were placed with males in the evening when in heat, estrous being determined by the vaginal smear method. The morning on which sperm were found in the vagina was considered as the first day of pregnancy. The rats were kept in an air-conditioned room (74-78°F). The regular diet (diet XIV), given dry, was supplemented after operation by a wet mash of diet I given daily.[‡] Lettuce was given 2 to 3 times weekly. On the 22nd day of pregnancy the rats were placed in individual cages and observed every few hours for the following 4 days. At autopsy

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[†] The authors would like to express their appreciation to Attaline McKnight for her skillful assistance in removal of the separate lobes of the pituitary.

[‡] Diet XIV consists of 68.5% wheat, 5% casein, 10% fish meal, 10% alfalfa leaf meal, 5% fish oil, 1.5% NaCl, KI added (analysis 1 μg iodine per g diet). Diet I consists of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil, 1.0% fish oil, 0.75% NaCl, 1.5% $CaCO_3$, KI added (analysis 1 μg iodine per g diet).

TABLE I. Effects on Parturition of Post-Operative Terramycin Treatment in Completely Hypophysectomized Rats.

	No. of animals			Parturition, %	
		Parturition			
Treatment	Total	Normal	Abnormal	Normal	Abnormal
Terramycin	30	12	18	40	60
No terramycin	27	5	22	18	82

TABLE II. Comparison of Effects on Parturition of Removal on the 12th Day of Gestation of the Complete Hypophysis or Its Separate Lobes. (Terramycin given post-operatively.)

	No. of rats			Parturition, %	
		Parturition			
Type of operation	Total	Normal	Abnormal	Normal	Abnormal
Post. lobe removal	10	9	1	90	10
Ant. " "	12	3	9	25	75
Total hypophysectomy	16	4	12	25	75

on day 26 the pituitary site was carefully inspected under a dissecting microscope. The few animals in which there was doubt as to the type or completeness of the operation were discarded. In order to confirm the presence or absence of anterior lobe tissue the mammary glands, ovaries, adrenals, and thyroids were removed and fixed in Bouin's fluid, imbedded in nitrocellulose, sectioned at 7 μ, and stained with hematoxylin and eosin. The condition of the lungs was also checked as possibly pertinent to the question of the resistance of the hypophysectomized rat to the strain of prolonged gestation and parturition. The animals were classified in one of two categories depending on the duration and mode of termination of pregnancy: 1) normal, which included the animals which gave birth at term to living young, without prolonged labor; and 2) abnormal, which included the animals which died shortly before, at, or after term; those which resorbed; and those in which gestation was prolonged by 1 to 3 days.

Results. Effects of terramycin treatment on parturition in completely hypophysectomized rats. Terramycin, injected daily for 2 days post-operatively, definitely increased the number of animals surviving hypophysectomy; 25% of the animals died in the immediate post-operative period if no terramycin was administered, whereas only 5% died when the antibiotic was given.§ More unexpectedly, there were also increased numbers of survivors after normal parturition in the group receiving terramycin (Table I).

Effects of ablation of whole pituitary and of separate lobes on parturition. When the posterior and intermediate lobes were removed 3 to 7 weeks before the animals were bred, 6 of 7 rats delivered on time (22 to 23 days) without complications. They lactated well. (The seventh animal in the group also appeared to have littered as judged from autopsy evidence.) When the posterior and intermediate lobes were removed on the 12th day of pregnancy, leaving the anterior lobe intact, normal parturition, followed by lactation, took place in 9 out of 10 animals; only one animal delivered late, on the morning of the 24th day. Rats in which the anterior lobe only had been removed on the 12th day behaved in general as did the completely hypophysectomized rats. From a group of 12 such animals 3 had a normal parturition, while in 9 pregnancy and parturition were abnormal. The same ratio existed among rats from which the total pituitary had been removed, 4 out of 16 delivering normally. No difference could be detected histologically between the endocrine organs of rats which delivered normally and those in which the duration of pregnancy and parturition were abnormal.

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§ Mortality after hypophysectomy is higher in adult, pregnant rats than in immature rats. (In rats hypophysectomized at 26-28 days of age the mortality in the first 10 days post-operative is nearer 6% and is reduced to practically zero after comparable treatment with terramycin.) In these experiments where adults were used it was not possible to select as carefully the animals to be hypophysectomized due to the more limited number from which to choose; also the operative procedure required more time than in younger animals with thinner bony plates. The operations where special lobes were removed also required more care.

Discussion. There are certain differences between these results and those previously reported in the literature on the effect of the pituitary on parturition, as shown by its removal. Some animals from which either the complete hypophysis or the anterior lobe were removed, littered with slight or no delay and gave birth to living young. In animals which had not yet delivered on the 26th day after conception, no living young were born or were found alive *in utero* at autopsy. Typically, at this late period, the young were macerated and the uterus was filled with blood; resorption of some of the dead fetuses had begun. Teel(5) found that in prolonged pregnancy induced in intact rats by administration of pituitary extracts, living young could be removed from the uterus on the 25th day but that all fetuses were dead by the 26th day. Selye, *et al.*(3) reported that hypophysectomized rats gave birth to living young as late as the 26th day. It is possible that the use of different strains of rats, or of a different method of designating day 1 of pregnancy may be responsible for this difference in results. Pencharz and Long (1,2) reported that pregnancy was prolonged and that the animals died without giving birth following hypophysectomy on the 11th to 20th day of pregnancy. In the studies here reported parturition was normal in some of the rats.

Terramycin injections given post-operatively not only reduced the fatality rate after the operation, but also unexpectedly increased the number of normal births. Furthermore, among animals with abnormally prolonged pregnancy, the fatality during parturition was lower than it was in non-injected animals. Whereas Pencharz and Long(2) reported that 71% of 28 hypophysectomized animals died after unsuccessfully attempting to deliver, in this study deaths were recorded in only 7% of 30 animals treated with terramycin, and in 37% of 27 non-injected animals.

Ablation of the posterior lobe, either before pregnancy or on the 12th day of pregnancy, did not interfere with normal gestation parturition, or onset of lactation. It may be claimed that the infundibular stalk and the tuber cinereum were left intact and thus enough of the neurophophysis remained to secrete amounts of oxytocin sufficient for normal parturition. These parts of the neurophophysis, however, also remained when complete hypophysectomy was performed, yet delivery was abnormal in most cases. Furthermore, removal of the anterior lobe was followed by deranged parturition even though the posterior lobe as well as the stalk was left intact. These findings, then, confirm the previous observations that the posterior lobe is not essential for parturition in the rat.

The effects on parturition of removal of the anterior lobe were the same as those which were caused by total hypophysectomy. In both cases, most of the animals (75%) delivered late, failed to deliver and began to resorb the fetuses, or died. It seems convincing that the derangement in the birth mechanism occurring after total hypophsectomy is due to the removal of the anterior lobe. It is not known, however, whether this essentiality of the anterior lobe is due to a direct or an indirect influence on the process of parturition. A direct action is not conclusively proven, and there are indications that metabolic or other factors indirectly related to anterior pituitary function are involved. The fact that some animals in each experimental group delivered normally, and also the variation in the severity of the derangement in the animals delivering abnormally, indicates that a multiplicity of factors influence parturition. Among the factors which may interfere with the process are the state of health of the animals, derangement in carbohydrate metabolism, decreased food consumption, weight of the fetuses, and the persistence of corpus luteum function.

The general health of the animals may be of great importance in determining the ability to expel the fetus. Hypophysectomized animals are very susceptible to respiratory infections. Many of the animals which failed to deliver normally had pulmonary lesions, though this was not limited to them alone. The finding that terramycin injections following hypophysectomy reduced not only the postoperative fatalities but also the fatalities during parturition, and resulted in a higher rate of normal deliveries, may be explained in part as due to reduction of pulmonary infections. After hypophysectomy the carbohydrate metabolism of rats is well known to be deranged. Though it is adequate for life under favorable conditions there is small margin for meeting adverse conditions. Such animals while attempting to deliver may soon exhaust their glycogen supply and pass into a hypoglycemic shock, dying if parturition is prolonged. The food consumption is less than normal in pregnant hypophysectomized rats(6). In these animals, which are already susceptible to hypoglycemia, the decreased food intake may further contribute to the exhaustion, failure to give birth at term, and eventual death.

The weight of the fetuses on the 21st day of pregnancy in hypophysectomized rats is reported to be the same as or smaller than the weight of fetuses from normal rats(6,7). The size of the fetuses would then not be a primary factor in preventing parturition at term. However, when parturition is delayed, the size of the fetuses may continue to increase which may then secondarily cause difficulties in late delivery. No direct evidence is available regarding this point in hypophysectomized rats. One can only reason from normal pregnant rats in which the fetuses become larger than normal young at term when gestation is prolonged by injections of anterior lobe or luteal extracts. (5,8).

That the corpora lutea of hypophysectomized pregnant rats continue to function is suggested by their morphology. Pencharz and Long(2) noted that the corporea lutea were indistinguishable from those in normal rats at the same day of pregnancy, Further, it was observed in the course of this study that at autopsy on the 26th day the corpora lutea of rats hypophysectomized on day 12 were larger than those of normal post-partum rats. This suggests the continued secretion of a leutotrophic factor, which, in the absence of the pituitary, must have been provided by the placenta. Astwood and Greep(9) have demonstrated such a luteotrophic factor in placental tissue.

Another mechanism involved in the prolonged life of the corpora lutea which must not be overlooked is the possible removal with the pituitary of a corporolytic factor(10) which in the normal animal would act to diminish corpus luteum function. This subject has not yet received sufficient attention.

Summary. 1. Ablation of posterior and intermediate lobes of the hypophysis either before conception, or on 12th day of gestation resulted in no disturbances in duration of pregnancy, in parturition or in onset of dactation. 2. Removal of anterior lobe of the hypophysis had the same effects on pregnancy and parturition as did total hypophysectomy. After both types of operation 25% of the rats delivered normally at term; 75% had a prolonged pregnancy (1 to 3 days) and either delivered late, resorbed, or died in an effort to deliver. 3. Terramycin injections given on the first and second days following hypophysectomy, decreased post-operative mortality, resulted in a larger percentage of normal deliveries, and in a smaller fatality rate at parturition as compared with non-injected animals.

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A Self-Guiding Catheter for Cardiac and Pulmonary Arterial Catheterization and Occlusion.[*] (20745)

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In closed-chest dog preparations, catheterizations of the right heart and pulmonary arteries are usually accomplished with radio-opaque catheters which are placed by manipulation with fluoroscopic visualization(1). The catheter described below enjoys certain advantages in that it can be placed quite rapidly without apparent trauma to the heart and vessels, it can be easily constructed and above all, it does not require a fluoroscope for placement. It is particularly suited to experimental procedures where pressure recording and blood sampling are desired. Furthermore, it may also be used to block the blood flow at some particular site.

This catheter has been used routinely in this laboratory for two years. Its success is due mainly to its large degree of flexibility whereby a small inflatable balloon located at its tip is able to guide the catheter automatically in the direction of the blood flow from the veins through the right heart and into the pulmonary artery.

Construction of Catheter. The catheter (Fig. 1) is approximately 3 feet long and consists of 2 tubes. The outer tube, through which pressures are recorded or blood samples are withdrawn, consists of polyvinyl[†] (O.D. 3.2 mm; I.D. 2.4mm). The other tube consists of polyethylene[‡] (O.D. 1.27 mm; I.D. 0.86 mm) and is placed inside. It emerges through the wall of the larger tube at each end where it is secured by thread as indicated in the figure. This tube serves to inflate the balloon which consists of the tip of a rubber "finger-cot", likewise fastened by thread after first flame-flanging the polyethylene tip. The thread-covered areas are coated with liquid latex and are vulcanized by a 20-second exposure to the vapors of sulfur monochloride. A No. 13 gauge needle is fitted into the lumen of the larger tube and a No. 20 gauge needle serves the smaller tube for attachment to a three-way stop cock and syringe, respectively.

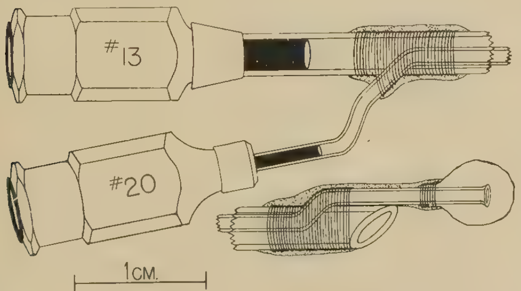

FIG. 1.
Detailed construction of a self-guiding catheter.

Placement procedure. The above catheter size serves adequately for dogs of 20 kg or more. The larger tube is filled with saline and connected to either a gravity-feed, heparinized-saline drip or a saline manometer by means of a 3-way stop cock. The smaller tube is attached to a 10 cc syringe which also contains saline. The catheter is introduced into the jugular vein with a balloon in a deflated state. The 3-way stop cock is then turned to record pressure in the manometer via the larger tube. The manometer is set to zero with reference to venous pressure in the vicinity of the heart. One cc or more of saline is then injected into the balloon. As the catheter is slowly advanced into the jugular vein, the partially inflated balloon, acting like a sail, is swept into the right heart by the blood flow. The entrance of the balloon into the right ventricle is signalled on the manometer by a rise in pressure from zero to that of mean right ventricular pressure. This is usually about 10 cm H_2O. With the balloon still inflated, further slow admittance of the catheter allows the balloon to be swept into the pulmonary arterial system. This event is signalled by a further rise in pressure to approximately double that of the mean right ventricular pressure. The balloon can now be deflated and the three-way stop cock turned from the manometer to the saline drip to prevent clotting. The entire catheterization procedure takes approximately 5 minutes. The maximum time of passage through the right heart is usually no more than a few seconds.

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[*] This work was supported by the Air Research and Development Command, Wright Field.

[†] No. 12 transflex extmold plastic tubing—Irvington Varnish and Insulation Co., Irvington, N. J.

[‡] Tubing PE-90-Clay-Adams Co. Inc., 141 E. 25th St., New York City.

For acute occlusion of the pulmonary arteries or the chambers of the right heart, the balloon may be reexpanded. Right or left pulmonary artery occlusion is usually obtained with 5-7 cc of saline. If specific placement of the balloon is required, this system may be filled with a radio-opaque liquid instead of saline and placed by means of fluoroscopy.

Discussion. The success with which these catheters are placed is due mainly to their complete flexibility. In contrast to the usual stiff radio-opaque catheters, these cannot be "guided" at all and depend entirely for their directional movement on the flow of blood pushing the partially inflated balloon through the chambers of the heart—hence "self-guiding". It should therefore be emphasized that the much more flexible polyvinyl plastic is necessary and that substitution of the stiffer polyethylene plastic for the outside tube is not recommended.

If the catheter is truly self-guiding, no skill should be necessary for its placement once it has been inserted into the jugular vein. This has been repeatedly verified by asking medical students and other staff members to place this catheter into the pulmonary artery merely by watching the pressure rise in the saline manometer as they slowly advance the catheter into the jugular vein. The branching of the two main pulmonary arteries is such that this catheter will in most instances enter the left pulmonary artery.

One disadvantage of this catheter is the clots which frequently develop along the folds of the balloon. These, however, are minimal in size and number and have not interfered with the experimental procedures.

Summary. 1. A self-guiding catheter has been described for placement in the right heart or pulmonary arteries of dogs. 2. This technic does not require a fluoroscope and localization is determined from the pressure recording. 3. The same catheter has likewise been used routinely for occlusion of the left pulmonary artery.

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Influence of Colchicine on the Form of Skeletal Muscle in Tissue Culture.* (20746)

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When mature mammalian striated skeletal muscle is cultured *in vitro,* as shown by Pogogeff and Murray(1), there ensues intensive regeneration of muscle with the formation of long continuous sarcoblastic ribbons or fibers. In the rat these may reach lengths approaching 3 mm, and widths of 6 to 15 μ (Fig. 1). Their cytoplasm is highly refractile, firm, and viscous or gelated(2); they exhibit a faint positive birefringence; they often show strong contractile activity (fibrillation and pulsation) and many subsequently differentiate, developing longitudinal and cross-striations.

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† Fellow of the Damon Runyon Memorial Fund.

FIG. 1. Control culture, at 17 days. Long slender sarcoblastic fibers. × 100.

In the course of investigations of the mechanisms of nuclear division in sarcoblasts, tissue cultures were treated with colchicine and certain other agents, The remarkable change in the form and structure of the muscle ribbons, leading to their dismemberment, which was observed consequent on administration of colchicine is the subject of this communication.

Materials and methods. A total of 210 successful cultures were prepared by explanting gluteal and hamstring muscle of suckling rats by the double coverslip method of Maximow (3). The planting mixture consisted of 1 part chicken plasma, 2 parts human placental serum and 1 part 50% chick embryo extract. Cultures were washed in Simms X-7 balanced salt solution(4) and fed thrice weekly with a mixture in which the plasma of the planting mixture was replaced by ox-serum ultrafiltrate. In subsequent feedings the proportion of embryo-extract was gradually somewhat reduced, to encourage differentiation. Cultures were maintained for periods of time ranging from 15 to 75 days, and two were kept for 185 days. At various ages, groups of 2 to 10 cultures were treated with colchicine or other biologically active substances, included in the feeding mixture to the final concentrations given in the following list: *Colchicine,* from 10^{-5} M in 5-fold dilutions to 10^{-8} M; chloral hydrate, 10^{-3} and 10^{-4} M; benzoquinone, 10^{-3} and 10^{-6} M; sodium barbital, 10^{-5} M; heparin 0.25 mg/ml, quinine dihydrochloride, 2.5 x 10^{-5} M; procaine base 4 x 10^{-5} M; "veratrine" 10^{-5} g per ml; d-tubocurarine hydrochloride, 10^{-5} M; "mapharsen" (3 amino 4 hydroxyphenylarsenoxide hydrochloride) 5 x 10^{-5} M. Untreated controls were also used, but it was thought that an assortment of other drugs which included mitotic poisons and agents which affect muscle contraction should be employed as further control material. The *standard test period* during which each agent was first allowed to act on a group of cultures was the usual interval between feeding, *i.e.* 48 hours. In subsequent experiments, periods of 12, 18, 24 and 36 hours and from 4 to 20 days of continuous exposure to colchicine were tested. Treatment was stopped by thorough washing in two changes of balanced salt solution; cultures were then either fixed, or re-fed and allowed to recover. The observations re-

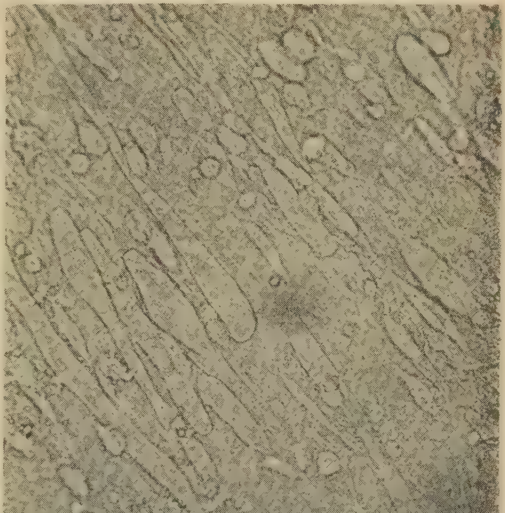

FIG. 2. Culture G 6, treated at age 15 days with 5×10^{-7} M colchicine. Short, widened segments 48 hr later. × 100.

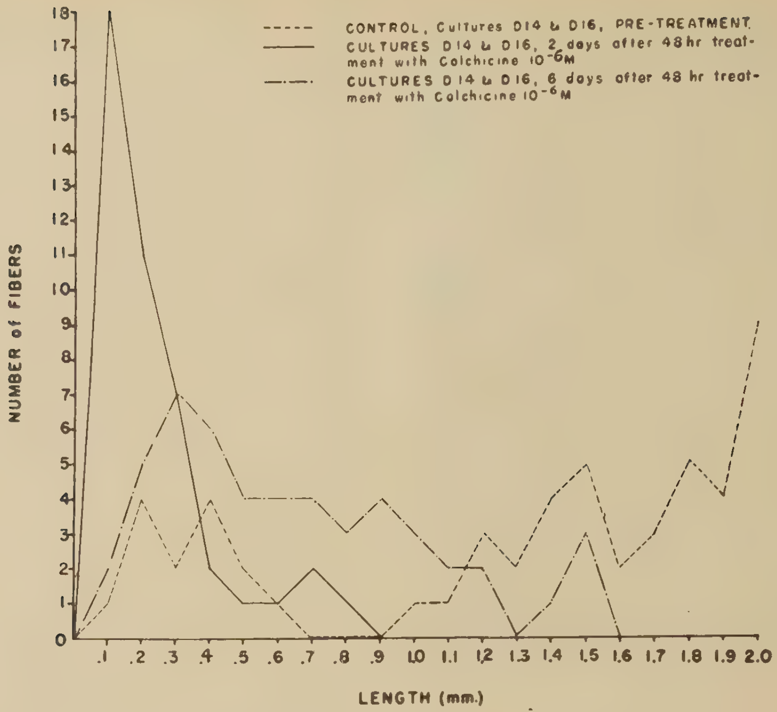

FIG. 3. Frequency polygon showing effect of colchicine on length of sarcoblast fibers at successive intervals. Note recovery phase 6 days after beginning of treatment.

corded in the present report were all made on living material.

Results. Untreated muscle cultures: Outgrowth of muscle slips was established in 7 to 15 days, and continued until the maximal rate was attained, usually in not less than 15-20 days from the time of appearance of the sarcoblasts. By this time some had attained lengths up to 3 mm. Measurements of the lengths of all muscle fibers in representative fields of the outgrowth zone, when plotted as a frequency polygon or histogram, were distributed in a characteristic bimodal curve, reflecting the presence of long, continuous fibers and lesser numbers of short strap forms of from 0.2 to 0.7 mm (Fig. 3).

Colchicine effects. Such cultures, when treated with colchicine in effective dosage, invariably suffered the same striking disruptive changes. At first, the fibers or straps developed focal expansions, ovoid or fusiform bulges, sometimes appearing somewhat haustrated. As these were pulled apart by their shortening and widening, they remained connected only by slender bridges of sarcoplasm. At the same time, sharp diagonal, transverse, tangential, and longitudinal clefts appeared in remaining lengths of the fibers, and effectively completed the process of segmentation which ultimately overtook almost all of the fibers. The developed picture was one of a generalized fragmentation of the muscle slips into short, distinctly widened, somewhat angulated segments, usually still in the linear disposition of their original fibers, and averaging 0.1 to 0.3 mm in length. As the effect of colchicine progressed, the pieces shortened and spread further, most measuring between 0.03 and 0.15 mm in length and 20-24 μ across (about three times the median widths of fibers in control cultures of the same age). They appeared as chunky, separated, sessile masses with rounded contours (Fig. 2), of definitely increased transparency and diminished refractility, usually without vestiges of striation, and apparently of much diminished consistency. It is of particular interest that some rhythmically contracting fibers, usually striated, continued to fibrillate even after segmentation was fairly advanced, the partially separated pieces contracting independently. In the later stages, as the effects of colchicine progressed, contractility was lost. Granules and droplets were not generally prominent, and all except the smallest segments contained one or more nuclei. The latter, typically elongated in the normal sarcoblast fiber, assumed a spherical shape with augmented diameter in the more advanced colchicinized segments, as though released from the constraint of the stiffened sarcoplasm. Anucleate fragments (sequestra) and a few of the other segments degenerated and disappeared. Most segments, however, remained viable, with relatively clear, transparent cytoplasm, or showed only minor toxic effects, even after prolonged continuous exposure to colchicine; for when the drug was removed they regenerated to nearly normal appearance and function.

Colchicine solutions in dilution to about 10^{-8} M elicited the change in the fiber form during the standard 48-hour exposure. In assessing dosage and effects, the principal variants considered were drug concentration, duration of action and age of the culture, or more precisely, the size and degree of differentiation of sarcoblasts. In summary, it may be said that except with the lowest effective concentrations of the drug, the first changes were observed 5 to 9 hours after colchicine administration. With 36- to 48-hour exposures, they reached their maximal extent in 4 to 5 days after administration, regardless of whether the culture was continuously colchicinized, or whether it had been washed and fed after the exposure period; that is, the effect of colchicine continued after it was removed from the medium.

In the course of 18-36 hours exposure to effective concentrations of colchicine, almost all of the slender and more undifferentiated sarcoblastic ribbons segmented; the broader, more differentiated fibers remained as a rule in earlier stages of colchicine alteration, being apparently more resistant to the drug. Cultures of intermediate or older age group, whose population of more differentiated and larger elements was greater, were accordingly slower to respond.

Concentrations of colchicine of 10^{-5} M were somewhat toxic to some cultures; the magnitude of the effects elicited by lower titers on fibers of similar ages was proportional to dosage: concentration x time of exposure.

Recovery. The rapidity and completeness of recovery were inversely proportional to drug dosage and toxicity. After a standard 48-hour treatment with colchicine concentrations of 10^{-5} M or less, recovery occurred in most cultures within a fortnight. Restitution did not occur in the presence of colchicine.

Controls. Changes of the kind described were never seen in the fibers of normally growing untreated muscle cultures. Neither the mitotic poisons nor the alkaloids, nor the organic arsenical listed, administered in concentrations comparable to or greater than the colchicine concentrations, produced an effect in any way similar to that of colchicine. These effects of colchicine on sarcoblastic ribbons were unfailing, and elicited specifically by colchicine, in high dilution.

Discussion. Three points of interest emerge from these observations of the effects of colchicine upon the sarcoblastic cytoplasm: the low concentrations at which they may take place, the length of time required for full effect and for recovery, and the dramatic nature of the changes themselves.

It is apparent that colchicine exerts a profound effect on the structural integrity of the immature muscle fiber, in concentrations considerably below those which inhibit mitosis in fibroblasts of the same species. It is probably significant that concentrations of the same order (about 2.5×10^{-8} M), according to Barany and Palis(5), condition a 25-30% decrease in the rate of the viscosity fall of actomyosin gels induced by ATP. These small concentrations are supposedly without visible effect on adult "muscle fibers"(5,6), although somewhat greater quantities are said to cause the Lundsgaard effect to appear in muscle preparations, owing to an interference with glycolysis(6).

Minimal effective concentrations of colchicine acting reversibly as a mitotic poison on cultures or isolated cells vary in different cell types, but usually exceed 10^{-6} molar. In the case of rat fibroblasts it is about 2×10^{-6} molar (7). But such concentrations may cause other effects such as alterations of cytoplasmic viscosity. Visual evidence of decreased protoplasmic viscosity in resting cells treated with colchicine has been recorded(7-10). Colchicine causes Arbacia eggs to stratify more easily when centrifuged, and inhibits the increased gelation that normally occurs during their division or after fertilization(11,12). The reduced cytoplasmic viscosity caused by colchicine in onion root-tip cells is thought to be responsible for "c-tumor" formation(13). Stalfelt(14) however has reported that colchicine increases protoplasmic viscosity.

In regeneration *in vivo* and *in vitro,* skeletal muscle is one of the slowest-growing of the tissue types and is characterized by an extraordinarily long lag-period, in these observations 7-15 days for the rat, with an added 15-20 days before maximal growth is reached. It may be presumed that one factor in this characteristic slow growth is the elaborate differentiation which is proceeding simultaneously. It is interesting that although colchicine may be assumed to penetrate the cell very quickly, since the first changes are noted within 5-9 hours after exposure, its maximal effects are not reached for 4 or 5 days, and are exerted even though the agent might have been withdrawn after 36-48 hours. Recovery occurs in approximately a fortnight. In the rat fibroblast the mitotic effects of colchicine reach their height at 20 hours(7), and recovery occurs within an equivalent time.

It is reasonable to assume that the ribbon-like continuity of the sarcoblast fiber, its gelated condition and positive birefringence are explainable structurally in terms of a parallel arrangement of longitudinally oriented long-chain protein (myosin) micelles. This submicroscopic structural plan of the sarcoblast is re-exhibited in microscopic dimensions with differentiation and the development of longitudinal striation. It is possible that colchicine may interfere with the maintenance of this organized micellar pattern by acting to bring about either a folding of the long protein chains (fibrous to globular)(15) or a dissociation of aggregates of the polypetid micelles (13). The results of such action would be to effect a more random arrangement of protein chains, which might be grossly manifested in shortening, alteration of form and size, and decreased viscosity.

It is suggested that such action by colchicine is visibly expressed here in destruction of the ribbon continuity and in the shortening and expansion of resulting fragments of the sarcoblastic fiber, a protoplasmic mass in which the alignment, length and numbers of oriented protein chains reach extreme development.

Summary. Colchicine administered to tissue cultures of rat skeletal muscle in concentrations of 10^{-8} molar or greater causes a breaking-up of the sarcoblastic ribbons into short, widened segments of apparently decreased consistency. These segments remain viable. 9 other biologically active agents used as controls have no such effect. A possible mechanism of this action of colchicine is discussed.

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Influence of Cortisone on Nervous Tissue, Adrenal and Thyroid. (20747)

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A recent investigation by Castor *et al.*(1) indicated that the administration of cortisone to rats induces chromatolysis and cytoplasmic vacuolation of thalamic and hypothalamic nerve cells. The present study was designed primarily to determine whether the changes described by this group could be substantiated and to investigate the effects of large doses of cortisone on the histology of the cat brain. Furthermore, observations were recorded relating to the effects of this hormone on the adrenal and thyroid glands of the cat.

Materials and Methods. A) Cats: The 9 normal cats used in this experiment were subdivided into 3 groups. Group I consisted of 2 animals which received cortisone acetate* for 10 days at a daily dosage of 50 mg per kg of body weight. The 4 animals in Group II were given 25mg of cortisone acetate per kg of body weight daily for 10 days. Group III was comprised of 3 control cats to which was administered a daily injection of the vehicle for 10 days. A tenth cat was given the hormone for 20 days at a dosage of 25 mg per kg of body weight. All the injections were subcutaneous. One week in advance of the first injection all the cats were started on a controlled daily diet of 100 g of horsemeat and 100 ml milk, and maintained on this regimen until the experiment was terminated. Each animal was weighed daily. Twenty-four hours after the last injection the cats were sacrificed, their heads perfused with 10% formalin and the brains removed. After further fixation, the brains were embedded in nitrocellulose. The thyroids and adrenals of these animals were also removed, fixed in 10% formalin and embedded in paraffin. Frontal serial sections of the brains were cut at 10 μ; every tenth section was stained with cresyl violet, and the next section with toluidine blue. The thyroids and adrenals were cut at 6 μ and stained with Gomori's trichrome stain. B) Rats: The brains of 44 young adult Sprague-Dawley rats were used in this study. Twelve animals were given daily subcutaneous injections of 5 mg of cortisone for 20 days and pair-fed with 12 uninjected controls. Another group of 10 animals was given 8 mg of the hormone for 10 days, and pair-fed with 10 uninjected controls. Twenty-four hours after the last injection the brains were removed, fixed in 10% formalin and embedded in paraffin. Frontal serial sections were cut at 10 μ and stained with toluidine blue. Insofar as possible, control and experimental sections of all the nervous tissue were matched area for area and the slides stained in pairs. A systematic study of the entire brain was undertaken. The hypothalamic nuclei examined were those listed by Rioch, Wislocki, and O'Leary(2), and Ingram(3).

Results. A) Nervous system: Microscopic examination of the cat and rat brains revealed no evidence of cytological changes, such as chromatolysis or vacuolization. There were also no indications of glial proliferation, hyperemia, hemorrhage or edema of the brain or meninges. B) Adrenals: The glands of the treated cats all displayed a marked atrophy of the zona fasciculata and a slight regression of the zona glomerulosa. There were no demonstrable changes in the zona reticularis. C) Thyroids: The glands of the control cats displayed medium sized or large follicles which contained, in general, large quantities of dense colloid. The epithelial lining of these follicles was cuboidal or lower. The thyroid follicles of the cortisone-treated groups were noticeably smaller and contained little or no colloid. The cells lining the follicles were definitely taller than those of the control group.

Discussion. A) Adrenals. The cortical atrophy observed in the cats given cortisone is consistent with the findings of others in the rat, and much evidence has accrued to indicate that this involution is due to a restricted output of ACTH by the anterior pituitary (4-6). B) *Thyroids.* The morphological picture of the thyroid in the cortisone-treated cats appears to be indicative of a higher than normal level of thyrotrophic activation. This is in agreement with the findings of Higgins *et al.*(7), and Halmi and Barker(8) in rats. C) Nervous system: Castor *et al.*(1) found that ACTH given to rats in 10 mg dosages per day for a period of 10 days resulted in consistent cytological changes which were restricted to the cells of the hypothalamic paraventricular nucleus. Animals treated with similar quantities of cortisone showed changes of a more widespread nature which involved several hypothalamic and thalamic nuclei. The conclusion reached was that the changes effected by ACTH might be interpreted as evidence of altered secretory activity within the cells of the paraventricular nucleus. However, the ACTH used in their experiment was contaminated with posterior lobe hormones, and it is possible that the latter were responsible for the alterations in the paraventricular nuclei (9). Furthermore, the rats of the Castor group that were treated with cortisone were in poor condition at autopsy whereas the animals used in the present experiment were in excellent condition. This tends to favor the interpretation considered by the authors that the morphological changes in the central nervous system after cortisone treatment might possibly be non-specific and due to the general deterioration of the animals rather than to a specific action of cortisone on the nervous system.

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* Cortisone (Cortone acetate Merck) was generously supplied by Dr. Charles A. Winter, Merck & Co., Rahway, N. J.

Summary. 1. We attempted to ascertain the effects of large doses of cortisone on the histology of the cat and rat brain. In addition, the adrenals and thyroids of the cats were examined. 2. No change was encountered in the nervous tissue of cortisone-treated animals. 3. The adrenals of the cortisone-treated cats demonstrated the compensatory atrophy which is believed to be brought about by a decreased discharge of ACTH from the anterior pituitary. 4. The thyroids of the treated cats presented histological signs of thyrotrophic activation.

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Large Scale Preparation of Liver Mitochondria with the Waring Blendor.* (20748)

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(Introduced by Alexander L. Dounce.)

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For studies on the isolation of oxidative enzymes from liver, it was desirable to have a method for the preparation of mitochondria from fairly large amounts of tissue. Methods employing pestle type of homogenizers(1-6) are not convenient for this purpose. The Waring blendor can be used to disintegrate larger amounts of tissue, and procedures utilizing this means of homogenization have been used to prepare mitochondria from skeletal muscle(7), heart(8), and liver(9). However,

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preliminary investigation disclosed that the conditions of homogenization utilized in the first two methods were too drastic for liver and that complete disruption of the tissue did not occur under the conditions of the third procedure. Several workers(10-13) have claimed that good mitochondrial preparations cannot be obtained with the Waring blendor since a large portion of these cell bodies are damaged during the time required for complete rupture of the tissue.

A method is here reported for the production of virtually undamaged mitochondria from 50 to 100 g of liver with the aid of the Waring blendor. The first step in the procedure consists of a thorough maceration of the liver in a special tissue press (designed by A. L. Dounce and N. Naylor). The tissue is reduced to such a finely divided state that the subsequent time and motor speed required for homogenization in isotonic sucrose with the Waring blendor are not sufficient to damage the mitocondria. The latter are then isolated by differential centrifugation and washed twice with isotonic sucrose. Unlike the previous method for liver(9), the use of salt agglutination steps is avoided so that a clean cut separation of the mitochondria can be obtained. Proof that the mitochondria obtained by this method were nearly undamaged by the Waring blendor was obtained by comparison of the ATP-ase, fatty acid oxidase, and succinoxidase activities of these preparations with those of the mitochondria prepared with a pestle type homogenizer(14). Fresh "intact" mitochondria have virtually no ATP-ase activity(2,3,15). The ATP-ase activity is increased by aging the preparations(2,3,15) or by causing mechanical damage to the mitochondria(12). The fatty acid oxidase system, which is located in the mitochondria(1,16,17), is easily inactivated by conditions which damage these cell particulates(18-20). On the other hand, succinoxidase is more stable and remains relatively intact even in the particles which are formed by mechanical disruption of the mitochondria(10). Thus minor damage to the mitochondria would be revealed by a difference between the two types of preparations in respect to ATP-ase or fatty acid oxidase activity, whereas only gross damage would change the succinoxidase levels. Comparative experiments of this type have not heretofore been conducted with mitochondrial suspensions made with the Waring blendor (7-9).

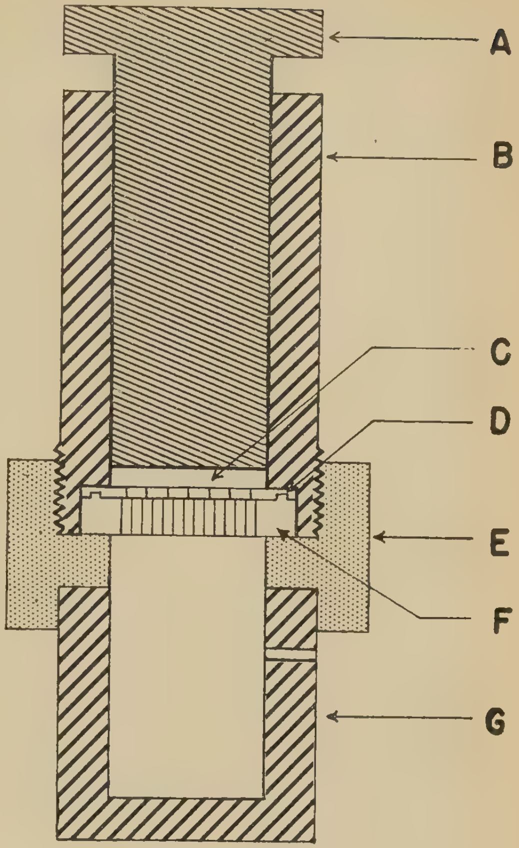

FIG. 1. Tissue press.

Preparation of mitochondria with the Waring blendor. Fifty to 60 g of fresh rat liver are thoroughly chilled in isotonic sucrose at –1°, and the tissue is then removed from the sucrose and pulped in the tissue press (Fig. 1) which has been chilled at 2° for 2 hours. The finely divided liver is weighed and then homogenized with 9 volumes (w/v) of isotonic sucrose at –1°, for 25 seconds at reduced motor speed in a chilled 1 liter Waring blendor cup. The voltage supplied to the motor, which draws 3 amperes, is reduced to 44 volts AC, or 1/3 of the usual amount. After the homogenate has been centrifuged at 1000 x *g* in 250 ml cups to remove the "nuclear fraction", the latter is washed with 150 ml of isotonic sucrose and resedimented at 1000 x *g*. The combined supernatants from the "nuclear fraction" are centrifuged at 10000 x *g* for 13 minutes in 50 ml centrifuge cups. The supernatant layer is poured off, and the resulting sediment is carefully layered with 2 ml of isotonic sucrose. The "fluffy" layer is loosend by gently swirling the tube and poured off. The remaining mitochondria are suspended in 500 ml of isotonic sucrose and resedimented at 10000 x *g*. The process of removing the "fluffy" layer, resuspending, and resedimenting of the mitochondria is repeated twice, and the latter are then suspended with the aid of a pestle type homogenizer(14) in isotonic sucrose of such volume that 1 ml of suspension contains the equivalent of 0.5 g of fresh liver. Centrifugation at 1000 x *g* is carried out with a refrigerated International centrifuge, whereas centrifugation at 10000 x *g* is brought about with a Servall centrifuge, type SS-1, which is operated in a cold room at 2°. The relative gravitational forces are calculated for the middle of the centrifuge cup. All solutions are kept at –1°, and the temperature should not rise to more than 3° during centrifugation. The motor speed and time of homogenization must not be varied from those given in the directions.

Other methods. The ATP-ase activity was estimated as the amount of orthophosphate (21,22) released per 10 minutes at 30° from 1 ml of reaction mixture containing 0.1 M sucrose, 0.015 M tris (hydroxymethyl) aminomethane, pH, 8.0, 0.003 M ATP, and 0.0075 M magnesium chloride. The assay was run with fresh mitochondria and also with preparations aged in sucrose at 30° for 20 minutes. The fatty acid oxidase or succinoxidase activity was measured by the rate of oxygen uptake at 30° with 0.001 M hexanoate or 0.01 M succinate respectively as substrates in 3 ml of a reaction mixture similar to that described by Lardy and Wellman(3). The phosphate acceptor system in the oxidase experiments consisted of creatine and a fraction of rabbit muscle extract precipitating between 50 and 70% saturation with ammonium sulfate(23). Air was the gas phase in these experiments. Dry weight was determined by heating at 105° for 10 hours after thorough dialysis of the suspension at 0° to remove sucrose. Mitochondria also were prepared with a pestle type homogenizer(14) by a modification of the method of Lardy and Wellman(3).

Tissue Press. In Fig. 1 is given a schematic drawing of the tissue press. The hollow cylinder B is 10.5 cm long and has an inner diameter of 3.9 cm and an outer diameter of 6.3 cm. The plunger A is turned to fit sleeve B closely. Disk C is 0.1 cm thick and is perforated with holes in groups of 3. Each group of holes is arranged in the form of an equilateral triangle with a 0.3 cm edge. Nine of these groups are contained in each 1.2 cm^2 of surface. They are bored in such a manner that they fit over holes 0.4 cm. in diameter on perforated disk D, when the two disks are aligned by means of the small pins E on plate D which project 0.1 cm into corresponding holes on plate C. One end of ring F is threaded to screw onto the bottom end of sleeve B, and the other end of F is recessed by boring so that it fits rather securely by slight friction on cup G. The latter is equipped with an air vent near the top 0.1 cm in diameter. All parts of the press are constructed of stainless steel. To assemble the press plates C and D are aligned by means of pins E and are inserted in the recess in B which has been bored out for this purpose. Then F is screwed onto B and the assembly is set onto G. After the tissue is placed in B, the plunger A is forced down by means of a Carver hydraulic press, and the macerated tissue is collected in G.

Results. Microscopic examination showed that the mitochondrial suspensions prepared with the aid of the Waring blendor were homogeneous and free of nuclei, whole cells, erythrocytes or cell debris.

In Table I it is shown that the succinoxidase, fatty acid oxidase, and ATP-ase activities of these mitochondria compare favorably to those prepared with a pestle-type of homogenizer. It should be noted that the enzymatic activity of the latter preparations is similar to that found by other workers(2,3,15). The ATP-ase activity of the fresh mitochondria prepared with the Waring blendor was usually slightly higher than that of preparations made with

TABLE I. Enzymatic Activity of Mitochondria Prepared in the Waring Blendor or in Pestle Type Homogenizer.

	Micromoles oxygen/10 min. /mg dry wt		ATPase as micromoles P/mg dry wt	
Preparation	Fatty acid oxidase	Succinoxidase	Fresh mitochondria	Mitochondria aged 20 min. at 30°
Waring blendor	.29	.92	.26	.61
" "	.32	1.0	.20	.66
Homogenizer	.33	1.1	.18	.70
"	.30	1.1	.16	.90

the mechanical homogenizer. In agreement with this observation was the finding (not given in Table I) that addition of the phosphate acceptor system was necessary for maximum rate of oxidation of hexanoate or succinate in the preparation made with the mechanical homogenizer but not in the mitochondria prepared with the Waring blendor.

Since disruption of the mitochondria is known to increase ATP-ase activity(24), it is possible that the blades of the Waring blendor do cause slight but measurable damage. On the other hand, the damage cannot be extensive, for the ATP-ase activity of both types of preparations was increased on aging the mitochondria, and the fatty acid oxidase activities of the two preparations were similar.

It thus appears that a rapid and convenient method for the preparation of mitochondria of high enzymatic activity from large amounts of liver has been developed. The procedure should be of particular value to those who wish to utilize the mitochondrial preparations as starting materials for enzyme preparations or to study stable mitochondrial enzyme systems.

Summary. A method is described for the preparation of mitochondria from 50 to 100 g of liver. The tissue is pulped in a special press and homogenized at a reduced motor speed for a short time in the Waring blendor in isotonic sucrose. The mitochondria are isolated by differential centrifugation from isotonic sucrose. The ATP-ase, fatty acid oxidase, and succinoxidase activities of these preparations were shown to be similar to those of mitochondria prepared with a pestle type homogenizer.

The authors wish to express their thanks to Dr. Alexander L. Dounce for the use of his tissue press.

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Effect of Anti-Hyaluronidase Compounds upon the Response of the Rat to Pituitary Trophic Hormones. (20749)

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The parenteral administration of aqueous solutions of protein hormones usually results in responses which are submaximal. This phenomenon has been ascribed to rapid absorption and dissipation from the injection site, increased excretion or inactivation. Attempts have been made to circumvent these suboptimal effects by combining these hormones with agents that will protect them from rapid absorption and/or inactivation. Preparations which have decreased the rate of absorption or dissipation of protein hormones have been oil (1,2), magnesium stearate pellets(3), colloids such as polyvinyl pyrrolidone, gelatin, acacia (4,5) and chemical adducts such as tannates (6).

Recently, Hamburger(7) and Cohen *et al.* (8) have described the use of anti-enzyme agents to enhance the activity of corticotrophin. Hamburger(7) used polyphloretin phosphate and attributed the enhancing effect primarily to the anti-proteolytic properties of the compound. Cohen *et al.*(8) used phosphorylated hesperidin and ascribed the enhancement to the anti-hyaluronidase activity of the material, since other substituted hesperidins (methyl chalcone) which had anti-proteolytic activity but no anti-hyaluronidase action did not enhance the activity of corticotrophin. It should be pointed out that the latter two groups of investigators used different corticotrophin preparations. Hamburger(7) employed a crude preparation, whereas the other group(8) administered an oxycellulose purified corticotrophin which has been shown to be relatively resistant to tissue inactivation.

The present communication deals with the effects of antihyaluronidase agents upon the action of exogenous pituitary gonadotrophin. Further observation on the effects of some of these compounds upon ACTH is also presented.

Material and methods. Immature female rats (Blue Spruce Farms) weighing 35-45 g were used. A purified preparation of pituitary gonadotrophin (Armour)* was incorporated into solutions of the substance to be tested. 0.5 ml injections were given subcutaneously twice daily for a period of 3½ days. On the fifth day the animals were sacrificed and their ovaries removed and weighed. The total dose of the gonadotrophin administered was 0.25 units and 1.0 unit (Armour). Hypophysectomized animals were also employed and consisted of immature female rats weighing 50-60 g. Hypophysectomy was accomplished by the parapharyngeal approach. They were injected 3 days after the removal of the pituitary. Since each substance was tested at a different time, FSH in distilled water was run concurrently with the FSH in the menstruum being tested. The following compounds were investigated for their effect on the activity of pituitary gonadotrophin. 1) 2% phosphorylated hesperidin† 2) 2.5% d-alpha tocopherol phosphate‡ 3) 1.25% d-alpha tocopherol phosphate 4) 5% Chondroitin sulfate 5) 10% Chondroitin sulfate 6) 3% sodium alginate 7) 5% hesperidin methyl chalcone. For the corticotrophin studies, hypophysectomized Sprague-Dawley male rats, 110-115 g body weight, were used 24 hours after surgery. Injections were given subcutaneously in 0.5 ml volume. Three hours after the injection the adrenals were removed and the adrenal ascorbic acid determined by the method of Mindlin & Butler(9). Purified corticotrophin (Princeton Laboratories), 25 U.S.P. units/mg was used.

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* The pituitary gonadotrophin was kindly furnished by Dr. Albert H. Holland, Armour Laboratories, Chicago, Ill.

† Phosphorylated hesperidin was kindly supplied by Dr. G. Martin, The National Drug Co., Philadelphia, Pa.

‡ Dr. S. Evert Svenson of Hoffman-LaRoche, Inc., made available the d-alpha tocopherol phosphate.

TABLE I. Effect of Anti-Hyaluronidase Compounds upon Response of Rat Ovary to Follicle Stimulating Hormone.

%	Vehicle	No. animals	Armour units	Ovarian wt, mg
	A. Intact animals			
.9	Distilled water	15	.25	16 ± 1
.9	” ”	21	1.0	40 ± 2
2.0	Hph	9	.25	23 ± 3
2.0	”	10	1.0	89 ± 13
.9	Distilled water	9	.25	20 ± 2
.9	” ”	14	1.0	46 ± 4
2.5	Tph	11	.25	64 ± 10
2.5	”	14	1.0	105 ± 13
2.5	”	3	0	12 ± 1
1.25	”	7	.25	45 ± 7
1.25	”	12	1.0	94 ± 12
.9	Distilled water	4	.25	12 ± 1
5.0	C.S.	8	.25	38 ± 5
10.0	”	5	.25	32 ± 10
3.0	A	5	.25	18 ± 2
2.0	H.m.c.	5	.25	12 ± 1
	B. Hypophysectomized animals			
.9	Distilled water	6	1.	41 ± 8
1.25	Tph	6	1.	80 ± 12
2.5	”	6	1.	85 ± 23

All values are means ± stand. error.

A = sodium alginate; Tph = d-alpha tocopherol phosphate; Hph = phosphorylated hesperidin; C.S. = Chondroitin sulfate; H.m.c. = hesperidin methyl chalcone.

Results and discussion. The data presented in Table I indicate that the activity of the follicle stimulating hormone when administered together with anti-hyaluronidase agents, was enhanced approximately 4 times as compared to the hormone activity of the gonadotrophin administered in distilled water. This is evident from the observation that 0.25 units of the gonadotrophin in conjunction with d-alpha tocopherol phosphate, phosphorylated hesperidin or chondroitin sulfate induced ovarian hypertrophy comparable to the response obtained with 1.0 unit of the hormone in distilled water.

All the compounds tested and found active in enhancing the effectiveness of the gonadotrophin were chosen because of their strong hyaluronidase-inhibiting properties(10-12). Since some of these substances also possess anti-proteolytic properties, it was deemed important to determine whether the enhanced activity was a result of delayed absorption due to the anti-diffusion property of the compounds, or to an inhibition of proteolytic destruction at the site of injection or in the blood stream. To separate these effects the methyl chalcone of hesperidin was employed. This compound possesses no anti-hyaluronidase activity(10) but does have considerable anti-proteolytic activity when tested *in vitro*(8). As may be observed from the data, this compound exerted no enhancing effect upon the exogenous FSH. This finding confirmed the hypothesis that the enhancing effect of the active compounds was due primarily to their anti-hyaluronidase properties. McShan & Meyer(13) had earlier described the augmenting effect of hemin upon sheep pituitary gonadotrophin, but offered no explanation for this phenomenon. Recently(14) hemin was found to possess a strong anti-hyaluronidase effect, which property may explain the earlier observations of McShan & Meyer(13).

Enhancement noted in the hypophysectomized animals with 1.25 and 2.5% d-alpha tocopherol phosphate indicated that the augmented ovarian weight so obtained could not be ascribed to increased endogenous pituitary gonadotrophic release or secretion. In addition, administration of the anti-hyaluronidase preparations without FSH failed to induce any gonadal stimulation, indicating that these agents do not possess inherent gonadotrophic activity or potentiality.

One should note that of the three anti-hyaluronidase agents used in this study and in the doses employed, phosphorylated hesperidin produced the greatest degree of local tissue reaction[§] while no reaction was noted after injection of the chondroitin sulfate menstruum. The local tissue irritative response to d-alpha tocopherol phosphate was less than that noted with phosphorylated hesperidin.

Chondroitin sulfate was of interest because it offered an opportunity to observe a compound which inhibits hyaluronidase by competitive inhibition(12). Its effect upon corticotrophin was also examined. The result obtained (Table II) indicated that it was effective in enhancing the activity of corticotrophin as well as FSH.

The augmented response noted after incorporating FSH or ACTH compounds with

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[§] This irritative reaction may be due to contaminants.

TABLE II. Effect of Chondroitin Sulfate upon ACTH Induced Adrenal Ascorbic Acid Depletion, 3 Hr Post Injection. 5 animals in each series.

%	Vehicle	Dose ACTH, U.S.P. units	Adrenal ascorbic acid, mg/100 g adrenal gland
.9	Saline	0	371 ± 10
.9	"	1.0	273 ± 10
8.0	C.S.	1.0	155 ± 4
16.0	Gelatin	.1	236 ± 17
8.0	C.S.	.1	248 ± 7

preparations inhibiting hyaluronidase directly or by competitive inhibition may be ascribed to decreased absorption of the trophic material from the injection site. That the decreased rate of absorption from the site of injection may play a part in augmentation of pituitary gonadotrophic hormone was shown by the studies of Meyer and Hertz(15). They demonstrated that hourly injection of pituitary gonadotrophins over a 4½-day period produced a marked augmentation of response over that noted when the same dose was given at 12-hour intervals.

Summary. Anti-hyaluronidase agents, *i.e.* phosphorylated hesperidin, d-alphatocopherol phosphate, and chrondroitin sulfate were shown to be capable of increasing the effectiveness of administered pituitary trophic hormones such as follicle stimulating hormone (FSH) and corticotrophin. A four-fold increase in effectiveness of FSH was attained when the hormone was administered together with the anti-hyaluronidase agents as compared to the injection of the gonadotrophin alone. A similar degree of enhancement was obtained with corticotrophin. The augmentation induced by the anti-hyaluronidase compounds is attributed to the decreased rate of absorption of the hormone from the site of injection.

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Effect of Metabolites on Accumulation of Citric Acid in Fluoroacetate-poisoned Rats.* (20750)

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This study was undertaken in connection with our work on the effect of Krebs cycle inhibitors and metabolites on the performance and metabolism of isolated dog hearts, (heart-lung preparations). On adding dicarboxylic acids of the Krebs cycle into the blood stream in such preparations the problem of permeability arose. It cannot be assumed *a priori* that such highly charged dicarboxylate ions as are normally formed and metabolized within the cell will diffuse into the cell as readily as the glucose molecule. To gain more information on this point we have undertaken to study the organs of small animals *in situ*, using in the experiments here reported the fluoroacetate

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poisoned rat, which appears particularly suitable for such a study, since here the Krebs cycle is blocked at the citric acid stage and the latter accumulates in some organs of the animal. This accumulation of citric acid, as Peters(1) points out, is the best evidence we have that the Krebs cycle functions *in vivo*. Potter(2,3) has utilized the fluoroacetate poisoned rat successfully to study metabolic pathways and what he termed their "sequential blocking" *in vivo*. Potter reasons, that if in addition to the fluoroacetate block one places another block at the succinate stage by injecting malonate for example, a decrease in the amount of accumulated citrate is to be expected. Such a decrease was observed by him in the kidney, thymus, brain, and spleen though not in the heart, and this constitutes further evidence for the functioning of the tricarboxylic acid cycle *in vivo*. Now if it is assumed that in a fluoroacetate-poisoned animal a considerable fraction of the available C_4 member of the Krebs cycle is trapped in the accumulated citrate, we can expect upon injection of each of these C_4 fragments, or their precursors, to observe an increase in citrate over and above that which is observed after injecting fluoroacetate alone, provided that the necessary amount of C_2 fragments is readily available. On the other hand, if an excess of the C_2 fragments or their precursors is injected, one may expect the excess of acetate to compete with fluoroacetate for coenzyme A, with a resulting decrease in fluorocitrate formation and hence a diminution in citrate accumulation. Although acetyl donors have been used as antidotes in fluoroacetate poisoning(4) no one appears to have demonstrated a decrease in the amount of accumulated citrate.

We have also performed preliminary experiments to test the effect of injecting the C_4 and also the C_2 members of the Krebs cycle and their precursors on the tissue level of citric acid in non-poisoned animals. Here, however, no predictions could be made on the basis of existing knowledge.

Methods. Male and female albino rats 6 to 7 months old were used. Although these rats are of unrecorded strain, they have been thoroughly inbred for a long period of time at this University. The metabolites were injected in a standard dose of 2.25 millimoles per 100 g body wt in a volume of 2 or 4 ml. Larger doses were tried in each case when the results were inconclusive. The aqueous solution of the acid after being neutralized with NaOH was injected subcutaneously in several places in the back of the animal. Thirty minutes after administration of the metabolite, 3 mg sodium fluoroacetate per kg were injected intraperitoneally. Two hours after the fluoroacetate injection the animal was sacrificed and the heart and kidney quickly removed. The ventricles and whole kidneys were used for analysis, these tissues being homogenized in two volumes of ice-cold isotonic KCl and rehomogenized after addition of enough 10% trichloracetic acid to make the final dilution 5%. After centrifuging, the clear liquid was used for citric acid determination by the pentabromacetone method as modified by Natelson *et al.*(5). We used Mallinckrodt purified petroleum ether B.P. 30-75°C, and omitted the evaporation step. None of the metabolites we studied interfered with the accuracy of the method. Control experiments in which the same volume of saline was injected instead of metabolite were always run simultaneously.

Results. The results obtained in fluoroacetate-poisoned rats are summarized in Table I. It can be seen that the standard dose (2.25 millimoles per 100 g body weight) of each of the C_4 members of the Krebs cycle caused a marked increase in the accumulation of citric acid in the heart, but no significant increase in kidney citrate.. 1-Glutamate and 1-aspartate produced a slight but significant increase in heart citrate, on the other hand they produced a small but significant decrease in kidney citrate. Pyruvate was less effective than the C_4 fragments in increasing heart citrate but it caused a significant decrease in kidney citrate. Of the C_2 fragments studied, acetate and ethanol caused a marked decrease in both heart and kidney citrate. The most striking results were obtained with propionate and propanol. The latter produced low citrate values that approached those found in normal *i.e.* non-poisoned animals. Valerate also caused a marked reduction in the level of accumulated citrate. Butyrate on the other hand, caused

TABLE I. Citrate Accumulation in Heart and Kidney of Fluoroacetate-Treated Rats after Injection of Various Metabolites. Unless otherwise stated, 2.25 millimoles of the metabolites were injected per 100 g body wt.

Metabolite	No. of animals	Sex	Citrate in heart (μg/g wet tissue), mean ± S.E.‡	Citrate in kidney (μg/g wet tissue), mean ± S.E.
α-Ketoglutarate	3	♀	2030	1510
Saline controls	3	♀	893	1307
Succinate	10	♀	1700 ± 108	1490 ± 45
Saline controls	10	♀	954 ± 38	1417 ± 129
Malate	10	♀	1937 ± 67	1680 ± 64
Saline controls	10	♀	1039 ± 32	1529 ± 58
Oxalacetate	3	♀	1952	1736
Saline controls	3	♀	1068	1592
l-glutamate	10	♂	1173 ± 64	1226 ± 58
Saline controls	10	♂	960 ± 53	1561 ± 99
l-Aspartate	10	♀	1353 ± 65	1284 ± 80
Saline controls	10	♀	983 ± 48	1534 ± 55
Pyruvate	4	♀	1380 ± 108	889 ± 30
" *	7	♀	1919 ± 61	750 ± 51
Saline controls	7	♀	1013 ± 62	1473 ± 53
Acetate	4	♀	914 ± 57	961 ± 55
" *	10	♀	149 ± 23	416 ± 36
Ethanol	5	♂	541 ± 40	367 ± 48
" †	10	♀	340 ± 26	85 ± 8
Propionate	7	♂	31 ± 4	273 ± 66
n-Propanol	5	♂	19 ± 5	94 ± 10
n-Butyrate*	10	♂	1644 ± 137	963 ± 88
Saline controls	10	♂	960 ± 53	1560 ± 99
Acetoacetate	10	♀	1240 ± 59	974 ± 48
Saline controls	10	♀	1010 ± 48	1479 ± 61
n-Valerate	7	♂	319 ± 58	466 ± 95

* 4.5 millimoles/100 g body wt.

† 22.5 " " " .

‡ Stand. error = $\sqrt{\dfrac{\Sigma d^2}{n(n-1)}}$.

a rise in heart citrate but an almost equal lowering of kidney citrate. Acetoacetate behaved like butyrate in increasing the heart citrate and decreasing the kidney citrate.

Table II summarizes preliminary results obtained when metabolites are injected into normal *i.e.* non-poisoned animals. Here it is seen that all metabolites produce a substantial rise in kidney citrate, ethanol being the least effective. On the other hand, there was no significant increase in heart citrate except where the double dose of acetate or butyrate was used.

A comparison of Table II with Table I reveals that the heart and kidney citrate levels obtained after injection of the double acetate dose as well as the standard propionate dose, are almost identical in the poisoned and in the normal animals; the same is true of the kidney citrate values with ten times the standard dose of ethanol. This suggests that under these conditions the metabolites mentioned have completely overcome the fluoroacetate block.

Discussion. In interpreting results obtained after injecting various substances into a whole animal, certain factors must be considered which may play little or no part in the study of homogenates, tissue slices or isolated organs. For, if the reaction of one organ is different from that of another, it does not always follow that the metabolic patterns are different. Differences in cell permeability, and above all, in vascularity may be important. Furthermore, in an intact animal the kidney does not excrete all substances at the same rate, and, finally, a substance may be metabolized in one organ

TABLE II. Citrate Level in Heart and Kidney of Normal Rats after Injection of Various Metabolites. Unless otherwise stated, 2.25 millimoles of the metabolite were injected/100 g body wt.

Metabolite	No. of animals	Sex	Citrate in heart (μg/g wet tissue), mean ± S.E.	Citrate in kidney (μg/g wet tissue), mean ± S.E.
None	9	♂	19 ± 2.6	40 ± 1.4
Malate	4	♂	42 ± 9.2	389 ± 27
Pyruvate	4	♂	81 ± 29	224 ± 33
Acetate	4	♂	35 ± 3	102 ± 33
" *	4	♂	145 ± 19	527 ± 45
Ethanol†	5	♂	27 ± 5	70 ± 6.2
Propionate	4	♂	26 ± 1	192 ± 4
Butyrate	4	♂	51 ± 10	189 ± 21
" *	4	♂	326 ± 27	921 ± 101

* 4.5 millimoles/100 g body wt.
† 22.5 " " " .

and delivered to another organ in a changed form. This difficulty in interpretation, however, does not render experiments on the whole animal worthless. On the contrary, results so obtained, provide a stimulus for further experimentation using less complicated systems in an effort to analyze the importance of the various factors mentioned.

Fluoroacetate may penetrate organs to a varying extent. Hagan *et al.*(6) found that rat liver contained less organic fluoroacids after feeding fluoroacetate than kidney, heart and brain, although Potter's(2) bioassay revealed no significant differences between tissues. Now it is known that no citrate accumulates in the liver of fluoroacetate poisoned rats. In the light of our results concerning the inhibition of citrate accumulation in the heart and kidney by excess acetyl donors, could it be that this lack of citrate accumulation in the liver, the chief acetylating organ, may be due, in part at least, to the presence there of excess C_2 fragments?

The observed decrease in citrate accumulation in the kidney and heart of fluoroacetate poisoned rats after the administration of acetyl donors may be of toxicological interest. All the compounds that caused such an inhibition of citrate accumulation have been found to be effective as antidotes for fluoroacetate poisoning in one species or another(4). The question naturally arises as to whether this method may be utilized to screen compounds for use as antidotes for fluoroacetate poisoning. Kandel and Chenoweth(7) are of the opinion, however, that the blockade of the tricarboxylic acid cycle by fluoroacetate is not related to the development of symptoms of poisoning.

Why propionate and propanol are more effective than acetate and ethanol in inhibiting citrate accumulation in poisoned animals is not clear. Propionate is thought to be metabolized via pyruvate(8) but our results do not seem to support such a conclusion, since the effect of pyruvate is qualitatively and quantitatively different from that of propionate. The behavior of valerate is that which may be expected on the basis of β-oxidation to yield acetate and propionate.

The effect of the C_4 members of the Krebs cycle in increasing the citrate accumulation in the heart, but not in the kidney of fluoroacetate poisoned animals is difficult to explain. The injection of the C_2 or the C_4 members into non-poisoned animals causes a greater increase in kidney than in heart citrate level. Furthermore, the resting citrate level in the kidney is higher than in the heart. These facts could be explained by assuming that the metabolic wheel turns at a faster rate in the heart than in the kidney. The high kidney citrate value in fluoroacetate poisoning speaks against this hypothesis but this in turn might be explained by assuming that the kidney has a greater supply of C_4 fragments, perhaps by way of the corresponding amino acids.

Butyrate, pyruvate and acetoacetate increase the heart citrate value in poisoned animals, behaving in this respect like the C_4 members of the Krebs cycle. In the kidney, however, they reduce the citrate level, although not to the same extent as the C_2

fragments.

Summary. In fluoroacetate-poisoned rats, the C_4 members of the Krebs cycle further increase the accumulation of citrate in the heart but not in the kidney. C_2 members in appropriate doses may completely overcome the fluoroacetate block, a finding which may be of significance in antidotal therapy. In non-poisoned rats both C_4 and C_2 members of the cycle increase the citrate level in the kidney. The bearing of these findings on the metabolic patterns of various organs is discussed.

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Toxicity Studies on Hydroquinone.* (20751)

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A non-injurious chemical which significantly reduces the deterioration of food fats (oxidative rancidity) will be significant in reducing food deterioration, and in protecting human and animal health, since spoilage or rancidity of food fats destroys the palatability and the fat soluble vitamins in foods. The employment of hydroquinone as a food preservative has been advocated because of its effectiveness as an antioxidant(1-7). However, despite evidence to the contrary(8), the widespread consumption of this compound has not been considered safe(9). While hydroquinone (HQ) is definitely toxic at high dosages, this in itself does not necessarily militate against its use, providing the largest quantities that would be normally ingested prove innocuous. The purpose of this report is to present additional data on the chronic and acute toxicity of hydroquinone in rats, together with supplementary observations that were gathered on dogs, cats, and men.

Methods. Chronic experiments. Rats. Sprague Dawley male and female rats of weaning age (23 to 24 days old) were placed on a basic diet consisting of 23.7% dried skimmed milk, 9.24% lard, 66.0% ground whole wheat, and 1.0% iodized salt(10). Each kilogram of feed was supplemented with 4,800 international units of vit. A and 250 international units of vit. D. Fresh batches of food were prepared weekly and the rats were allowed to feed *ad lib.* Also, each animal received 5 g of fresh lettuce and 5 g of fresh lean beef each week. In Exp. 1, 4 groups of male and 4 groups of female rats, 10 rats in each of the 8 groups, were placed on the basic diet containing 0.0%, 0.1%, 0.5%, and 1.0% hydroquinone. In Exp. 2, the hydroquinone was heated together with the lard to 190°C for 30 minutes before incorporation into the food mix. Eight groups of rats, with 16 to 23 rats in each group, were fed the basic diet contain- 0.0%, 0.1%, 0.25%, and 0.5% hydroquinone. Finally, (Exp. 3) 8 groups of rats, with 20 rats in each group, were maintained on the basic diet containing both hydroquinone (0.0%, 0.1%, 0.5%, 1.0%) and citric acid (0.1%). The weights of the animals were recorded periodically for 103 weeks. After the first 6 months had elapsed, some of the males and females in Exp. 1, 2, and 3 were mated. The number of offspring for 2 successive litters was tabulated. At various periods during the experiment and at the end of 103 weeks, the following histological sections were prepared and examined: liver, omentum, kidney, spleen, heart, lung, bone marrow, stomach wall, pancreas, adrenal, subperitoneal and intramuscular abdominal fat. In addition to the studies initiated on immature animals, one group of 14 adult rats, together with an equal number of controls, were maintained for 9 weeks on the basic diet containing 5% hydroquinone.

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Dogs. Three female and 2 male mongrel pups 4 months of age from 2 litters were placed on "Pard" (Swift), supplemented by "Esbilac" (Borden). Two of the animals served as controls while one of the other 3 received 16 mg hydroquinone/kg/day for 80 weeks. The other two dogs ingested 1.6 mg hydroquinone/kg/day for 31 weeks and 40 mg/kg/day for the succeeding 49 weeks. Also, 5 adult male dogs received 100 mg/kg/day of hydroquinone for 26 weeks. The compound was administered to the animals in the form of sugar-coated tablets that were mixed in with the food. Routine analyses of the blood and urine were made periodically. After the experiment had terminated the animals were sacrificed and autopsied, and sections were prepared as described above.

Men. Two males ingested 500 mg of hydroquinone daily for 5 months, and 17 subjects, both male and female ingested 300 mg/day for 3 to 5 months. The compound was taken in 3 divided doses with meals. During a control period for one month and while the experiment was in progress, the following analyses were performed on the blood: % hemoglobin, hematocrit or red blood cell count, differential white blood cell count, sedimentation rate, platelet count, coagulation time and icteric index. Urinalysis included albumin, reducing sugars, white and red cell counts, casts and urobilinogen.

Acute experiments. Rats. The LD50 of hydroquinone was determined in Sprague-Dawley, Priestly, and Wistar rats. The compound, dissolved in glycerin, propylene glycol, or water, was administered in varying amounts by stomach tube. Animals that were fasted were deprived of food for at least 18 hours before the hydroquinone was administered. In each experiment 6 groups of 10 to 40 animals apiece were employed in each determination and the LD50 was calculated by the method of Bliss(11). *Dogs and cats.* The LD50 of hydroquinone was also determined in these two species. Twenty-eight unselected dogs and 10 cats were employed in this study and the compound was administered in sugar-coated tablets together with a small amount of meat.

TABLE I. Final Weights of Rats after 104 Weeks of Diets Containing Hydroquinone (HQ).

		% HQ in total diet				
	Sex	.0%	.1%	.25%	.5%	1.0%
H*	♂	527	534		460	467
	♀	300	379		300	248
H & citric acid	♂	502	401†		448	414†
	♀	273	326		320	271
H preheated with lard	♂	472	541	476	436	
	♀	280	269	263	226†	

* H = Hydroquinone.

† Significantly lower than controls at the 5% probability level.

Cumulative toxicity. Experiments. Rats. Six groups of 20 to 48 rats each were given doses of 500, 750, 1000, 1250, 1500, and 1750 mg of hydroquinone/kg by stomach tube 9 times in 12 days. An additional group of 16 animals received 500 mg/kg 101 times in 151 days. The temporal course of the fatalities was noted, and at the end of the experiment, the survivors were autopsied and histological sections were prepared as described above.

Results. Chronic Experiments. Rats. The final weights of the experimental animals after 2 years on diets containing up to 1.0% hydroquinone were generally not statistically different from the controls (Table I). However, during the first month of the experiment hydroquinone usually produced a definite decrement in the rate of growth of animals maintained on diets containing 0.5 and 1.0% hydroquinone. On the average, control males and females gained 27 and 22 g per week respectively, while rats reared on diets containing 1.0% HG gained only 16 g per week. This effect was not observed in the experiment where hydroquinone was previously heated with lard before incorporation into the diet. Hematological analyses of the experimental rats, including R.B.C., % hemoglobin, and differential W.B.C.

counts, disclosed no statistically significant deviations from the control values. The histopathological findings were also negative. The average number of off-spring of control and experimental females was almost identical for 2 successive litters. These offspring, reared on diets containing 0.0%, 0.1%, 0.25%, 0.50% hydroquinone that was previously heated with lard, grew at the normal rate. Adult rats maintained on the basic diet containing 5% hydroquinone suffered a 46% loss in weight over the course of 9 weeks, and developed aplastic anemia. Examination of the bone marrow revealed an average decrease of 66% in cellularity with marked atrophy of the hematopoietic elements. Pathological studies also revealed atrophy of the liver cord cells, lymphoid tissue of the spleen, adipose tissue, and striated muscle together with superficial ulceration and hemorrhage of the stomach mucosa.

Dogs. Pups that had received 1.6, 16, and 40 mg of hydroquinone/kg/day for 80 weeks grew normally, and adult dogs received 100 mg/kg/day for 26 weeks maintained their body weight. The gross autopsy findings, urinalyses, and hematological analyses were negative, and no pathological changes were observed in the experimental animals which could not be seen in the controls. However, hemosiderosis was usually more marked in the spleens, livers, and marrow of the control animals.

Man. Analyses of the urine and blood of 19 human subjects that ingested 300 to 500 mg of hydroquinone daily for 3 to 5 months revealed no abnormal findings.

Acute experiments. Rats. The LD50's of hydroquinone (approximately 1050 mg/kg) in different strains of rats treated under a variety of conditions is tabulated in Table II. Fasting the animal for 18 hours previous to the administration of the hydroquinone produced a 2- to 3-fold increase in the observed toxicity. Animals that survived 101 administrations of hydroquinone in 151 days, at a dose level of 500 mg/kg grew at the same rate as did the controls. Here again the autopsy findings were essentially negative, although more than half of the experimental animals succumbed after the first 2 months. In experiments where 6 groups of rats received from 500 to 1750 mg/kg of hydroquinone nine times in 12 days, it was found that 71% of the total mortality rate occurred within 24 hours after the initial dose. During the succeeding 11 days in which the remaining 8 administrations were made, the mortality rate averaged less than 5% per day. *Cats and dogs.* The LD50 of hydroquinone in dogs was found to be 299 mg/kg. Too few cats were employed to obtain a statistically accurate estimate of the LD50, but it apparently lay somewhere between 42 and 86 mg/kg.

TABLE II. LD_{50} of Hydroquinone (HQ) in Rats.

Strain	Carrier	Condition	LD_{50} (mg/kg) ± S.E.
Priestly	Glycerin	U*	1295 ± 2
	”	U	1005 ± 2
Sprague-Dawley	Propylene glycol	U	1090 ± 2
	Distilled water	U	1182 ± 2
	Glycerin	U	1081 ± 2
	Propylene glycol	F	323 ± 2
Wistar	” ”	U	731 ± 2
	” ”	F	298 ± 2

* U = Unfasted; F = Fasted.

Discussion. The LD50 is of little practical value for the estimation of the chronic and potentially cumulative toxicity of noxious substances, and the inferences of such a study are often misleading. For example, in fasted rats, dicumarol is less toxic than hydroquinone on an acute basis (LD50 of 310 mg/kg, in contrast to 540 mg/kg), while it produced an 80% mortality in animals maintained for 30 days on a diet containing .01% dicumarol(12). Assuming that each 250 g rat consumed 4 g of food daily, during the entire 30-day period each animal ingested less than 1/10 of the LD50 dose. Also, on the basis of the LD50, mapharsen was originally rejected in favor of salvarsan while it has since been demonstrated that the former drug produces far fewer delayed reactions than either arsphenamine or neoarsphenamine(13).

However, the acute determinations have divulged one fairly basic aspect of hydroquinone toxicity, *i.e.*, the increased susceptibility of fasted animals. The LD50 of unfed rats was 310 mg/kg in contrast to an average of 1064 mg/kg observed in normal animals. This finding is possibly related to both the enhanced rate of absorption of hydroquinone from the empty gastrointestinal tract and to the diminished detoxification potential of the fasted, glycogen-poor liver. In this connection it is interesting to note that the LD50 of hydroquinone when administered via stomach tube to fasted rats closely approximates the lethal dose observed when the compound is injected subcutaneously(14). Of course, if the chemical was ingested as part of the normal diet (as it would be if incorporated in food commercially), these considerations would not generally be applicable.

Rats subsisting for 2 years on diets containing from 0.1% to 1.0% hydroquinone generally grew and reproduced normally, although at the 0.5 and 1.0% levels there was a definite retardation in growth observed during the first month on the diet. Blood, tissue and urine analyses were negative. However, when adult animals were kept on a diet containing 5.0% of hydroquinone, a definite and progressive loss in body weight was observed together with generalized pathological alterations. The adverse findings were due at least in part to inanition, for these animals ingested less food. Hydroquinone has a discernable taste at this concentration in the diet. The results obtained with immature rats during the first month of the experiment may also be related to this observation, for when the hydroquinone was previously heated with the lard the animals grew at the normal rate.

Young dogs that received 1.6 to 40 mg hydroquinone kg/day for 80 weeks grew normally, and mature dogs that received 100 mg/kg/day for 26 weeks maintained their body weight. No consistent pathological findings could be demonstrated.

Contrary to reports in the literature(15), there was no evidence to indicate that hydroquinone possesses a cumulative toxicity, at least when given by stomach tube. At a dose of 500 mg/kg, (one-half the LD50), it was administered 9 times in 12 days to rats and the observed mortality rate was only 1.0% per day. Furthermore, rats which survived 101 administrations in 151 days grew at the same rate as the controls although more than half of the experimental rats had died over this period. The mortality rate was 0.4% per day.

The daily ingestion of 300 to 500 mg of hydroquinone for 3 to 5 months by 10 human subjects caused no observable alterations in the blood and urine. Since these quantities of accidentally ingested material have been reported to produce toxic symptoms(16,17), it may be significant to note that the compound was taken in 3 divided doses at meal time, so that the rate of absorption was undoubtedly diminished under these circumstances. This factor may also account for the absence of eye lesions in dogs that received 100 mg of hydroquinone/kg/day mixed with their food, such as were observed by Woodard(18).

It has been pointed out(2) that hydroquinone is widely distributed as a plant component. When ingested as part of the plant it has never been found to have toxic effects. Finally, it should be emphasized that the quantities of hydroquinone employed in these studies were relatively enormous. Assuming that a 70 kg man ingests one gram of fat per kilogram per day, and that all the fat was commercially treated with 0.01% hydroquinone, our human subjects did consume daily from 40 to 70 times the normal quantities of hydroquinone for 3 to 5 months, and on the same basis, our rats ingested daily up to 1000 times the calculated commercial dose. This tremendous safety factor demonstrated in these chronic studies strongly militates agains the exclusion of hydroquinone as a preservative in food fats.

Summary. Chronic toxicity experiments with hydroquinone have been carried out in rats, dogs, and men at dose levels greatly exceeding those to be expected if hydroquinone were utilized commercially as a preservative in food fats. It was found that relatively tremendous quantities of the substance had to be ingested before demonstrable physiological pathology occurred in the experimental animals. Acute studies on rats revealed that the LD50 of fasted animals was approximately one-third that of fed controls. The relatively large amount of hydroquinone that could be administered with impunity indicates that the compound could be used safely as a food preservative.

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Effect of N-Allyl Normorphine on Excretion of C-14-Labeled Morphine in Mice. (20752)

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The use of N-allyl normorphine in the treatment of respiratory depression resulting from overdosage of morphine or one of its synthetic substitutes(1,2) has been indicated by the many favorable responses which have been obtained both experimentally and clinically. The practical application of this antagonism, however, precedes any reasonable explanation of the mechanism involved in the spectacular recoveries reported in the literature. The experiments reported here indicate that N-Allyl normorphine is capable of producing at least two secondary effects in intact, morphinized animals, in addition to central stimulation. These results were obtained in the course of studies on the excretion of C-14-labeled, biosynthesized(3) morphine, utilizing small amounts of the drug; the quantity of alkaloid was not sufficient to cause central depression of remarkable degree.

Morphine has been shown to produce an antidiuretic response in experimental animals (4); the mouse shares this property of the alkaloid. This finding and other data suggested that a partial explanation of N-Allyl normorphine's action might rest in an increased ability to excrete morphine, free or conjugated, by activation of renal or other mechanisms, thereby decreasing the time that the drug could exert its effects.

Methods. Eight mice weighing 20 g each were injected intraperitoneally with 0.070 mg of C-14-labeled morphine base, each dose equivalent to 2100 counts (the specific activity of the compound isolated from Papaver somniferum was 30000 counts per mg per minute). Four of the animals were then injected intraperitoneally with 500 γ of N-allyl normorphine ("Nalline", Merck). The Straub reaction appeared in each animal approximately 2 minutes after injection of the C-14 morphine.

The urine was collected hourly in glass metabolism cages and suitable aliquots of each sample were plated on copper discs and counted in open window, gas-flow counters; results are expressed as counts recovered per hour. As the most significant findings appeared within 6 hours after injection, only

TABLE I. Excretion of C-14 Morphine, Counts/Hr.

	I		II		III		IV	
Hr	Morphine	Morphine and Nalline	Morphine	Morphine and Nalline	Morphine	Morphine and Nalline	Morphine	Morphine and Nalline
1	0	26	19	7	26	9	0	0
2	47	1580	11	766	11	1336	10	1036
3	28	0	375	43	13	0	0	28
4	1347	0	50	98	6	0	0	656
5	77	13	41	27	49	0	0	56
6	134	0	228	14	39	187	1189	164

TABLE II. Percentage of Dose Excreted.

	I		II		III		IV	
Hr	Morphine	Morphine and Nalline	Morphine	Morphine and Nalline	Morphine	Morphine and Nalline	Morphine	Morphine and Nalline
1-2	.022	76.5	.014	36.8	.017	64.0	.004	49.3
3-4	65.5	0	20.2	6.7	.9	0	0	32.6
5-6	10.0	.619	12.8	1.95	4.19	8.9	56.6	10.4

data obtained during this period will be presented here. The results of Table I and II were obtained in this study.

The data in Table I and II indicate that in each instance in which N-allyl normorphine was administered, a large portion of the injected dose appeared in the urine before the release of morphine by the control animal. These findings suggest that N-allyl normorphine produces in the morphinized animal effects in addition to the central stimulation for which it is well known. Such *secondary* effects could include(1) antagonism of antidiuretic hormone formation by the posterior pituitary or (2) facilitation of excretion by direct effect on the renal threshold. The antagonism by Nalline of morphine antidiuresis has been tested(5) and it has been found that Nalline significantly increases the volume of urine excreted by a group of morphine-treated animals as compared to morphinized controls. Other compounds have been found to antagonize the anti-diuretic action of morphine(5), and the mechanism of action of both these possible *secondary* effects is being studied further.

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Presence of Elastase in Teleost Islet Tissue.* (20753)

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Balo and Banga(1) recently described an enzyme, elastase, which solubilizes elastic tissue and is exclusively extractable from pancreas. Elastase, which is apparently specific for elastic tissue, acts upon the substrate without the release of peptides or amino acids. It appears to effect a conversion of this insoluble fibrous protein to a soluble globular form. Elastase has been effective in unravelling the chemical morphology of elastic fibers(2). Partially solubilized elastic fibers of horse ligamentum nuchae have been shown to contain fine, longitudinally oriented fibrils bound together by a matrix material of the same or similar composition. Morphologically, such partially solubilized elastic fibers closely resemble the frayed and fragmented elastic fibers generally found in arteriosclerotic vessels. Balo and Banga have reported that a markedly lower amount of elastase is present in the pancreases of arteriosclerotic than in normal individuals(3). Examination of their data indicates, however, that the fall in elastase activity is associated at least as much with age as with arteriosclerosis.

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There is some reason to question the assumption that elastase is a component of the acinar portion of the pancreas. The yield of elastase on extraction of whole pancreas is small compared to known acinar enzymes. Beef pancreas is a good source of elastase. Being a herbivore, it is difficult to picture a digestive function for elastase in this animal. Further, analysis of human pancreatic juice has not indicated the presence of elastase activity(4) and it is well known that elastic fibers pass through the alimentary tract with no apparent digestion. These, and other questions, led us to explore the possibility that elastase is a component of the islet tissue of the pancreas.

Methods. The teleost fish, *Lophius piscatorius,* is particularly suited for analysis of islet and acinar tissue of the pancreas since these two components are anatomically separate [Rennie(5); Macleod(6); McCormick (7)]. The islet tissue in Lophius exists as discrete nodules as much as 1 cm in diameter containing both alpha and beta cells(8). A distinct capsule is present with small islands of acinar tissue sandwiched between its layers of connective tissue. This capsule is readily removed by blunt dissection. Two large or principal islets and several smaller ones are present in the individual fish and are readily located in the mesenteries. Approximately 700 *Lophius piscatorius* (goose, monk, or angler fish) were collected at sea by a commercial dragger, placed on ice, and brought to the laboratory for removal of the islets. To check on a possible postmortem loss of elastase activity a limited number of newly caught fish were dissected at sea (by AIL) and the islets were stored in acetone for later fractionation. The bulk of the fish used varied in weight from 10 to 35 lb but a small number of small fish (1 to 10 lb) were used. No fewer than the two principal islets (1 cm in diameter) were collected and usually 2 to 4 of the smaller islets were large enough to be removed intact. After dehydration in acetone the connective tissue capsule was removed and stored separately. This capsule, as already indicated, includes small amounts of acinar tissue.

The pure islet tissue obtained after ecapsulation was ground to a fine powder with a mortar and pestle followed by a ground glass tissue homogenizer. Following established procedure(2) the islet powder was extracted with phosphate buffer at pH 6.0. This crude extract was either tested directly for elastase activity or else the elastase was salted out from it with $(NH_4)_2SO_4$ at 0.4 saturation. In the latter case, the salted out elastase was dissolved for use in carbonate buffer at pH 9.0 (optimum for elastase activity). The substrate was a standardized preparation of elastin from horse ligamentum nuchae(2). The elastase activity of fish islet powder and of acinar tissue in the islet capsule was compared with elastase prepared from equal amounts of mammalian pancreatic powder (Viokase, Viobin Corp.). Solubilization of the elastin fibers was observed with the aid of the compound microscope.

Mammalian elastin was readily solubilized by the extracts of the fish islets. Strong elastolytic activity was exhibited by samples extracted from as little as 100 mg of dried, defatted islet tissue. On the basis of rate of solubilization of elastin, the fish islet extract was more active than mammalian elastase prepared from equivalent amounts of mammalian pancreatin (Viokase). There was some difference in the course of action; although mammalian elastase does not begin to act on the elastin as rapidly as does the fish islet elastase, the former carries the solubilization of elastin to completion within a few hours while fish elastase may require as much as a few days.

Extracts prepared from the capsules of islets which contain acinar cells were completely inert with respect to elastolytic activity in so far as could be determined by the method of direct observation.

Discussion. In Lophius, it seems that elastase is confined to the islet tissue and that its mode of action differs somewhat from that of mammalian elastase. The latter may be due to species differences in the enzyme or the substrate, or both. The fact that elastase may be found in such divergent forms as fish and mammals indicates need for further study of the phylogenetic distribution of this enzyme and of a possible basic function for elastase. Experiments are currently being conducted to determine if elastase is also a component of the islet tissue of mammals.

Summary. Islet tissue of the teleost fish, *Lophius piscatorius*, contains elastase whereas acinar tissue seems to be free of this enzyme.

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Antigenic Differences Among Strains of Newcastle Disease Virus.* (20754)

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Newcastle disease, first recognized in the Dutch East Indies in 1926, is now known throughout the poultry raising regions of the world. Described in the Far East as a highly fatal infection of chickens, the virus evaded early recognition in many regions by appearing as a mild respiratory disease. In spite of the variations in pathogenicity and in other properties, most investigators have found that strains of the virus whether isolated in Asia, Africa or America are antigenically homogeneous(1,2). Contrary evidence is scanty and based on the relative immunogenicity of vaccines(3,4). The repository of strains of Newcastle disease virus assembled at the University of Wisconsin under the auspices of the Bureau of Animal Industry and the North Central Regional Technical Committee on Newcastle Disease provided material for an investigation of antigenic diversity among 13 strains of the virus. The cultures available had been isolated in many localities in the United States, Canada, and Mexico and from a few places in Europe and Australia over a period of 20 years.

Methods. The relative abilities of homologous and heterologous antisera to neutralize the embryo infectivity of the virus were compared. The immune sera were obtained from chickens and rabbits about 21 days after injection of virus and used unpooled. The rabbit serum was inactivated at 56°C for 30 minutes and chicken serum was used uninactivated. Infected allantoic fluids kept at –20°C were used as the virus source. Serial ten-fold dilutions of virus which had been incubated 30 minutes at room temperature with undiluted antiserum were inoculated intrallantoically in 0.1 ml amounts into 10- or 11-day embryonating eggs. Six eggs were used for each dilution, and all eggs candled twice daily for 7 days. The number of 50% lethal doses of virus neutralized by each serum was calculated by the procedure described by Smadel(5). On the basis of replicate tests prepared simultaneously by 2 individuals using the same stock virus and same serum, a difference of greater than one log was considered significant, 0.6 to 1.0 log of possible significance and 0.5 log or less of no significance (Table I). To keep the amount of non-specific difference at a minimum, heterologous serum-virus combinations were always compared to a homolo-

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TABLE I. Comparison of Serum Neutralization (SN) Titers Obtained by 2 Individuals Employing Same Stock Virus and Serum and Performing the Test on the Same Day.

Log SN titer

Virus	Serum	Test A	Test B
125-Iowa	125-Iowa	4.3	4.4
	11914 FD	3.3	3.5
	Naj	7.4	7.5
	RO	3.4	3.5
11914 FD	11914 FD	4.7	5.2
	Naj	5.0	5.0
	RO	4.7	4.7
	Vict	4.7	5.0

TABLE II. Cross Serum Neutralization (SN) Reactions with 5 Strains of Newcastle Disease Virus.

	Serum				
Virus	RO	Naj	Mil	125-Iowa	11914
RO		6.0–5.9* 7.9–6.7	6.6–4.8		6.4–6.7 6.0–6.7
Naj	6.7–3.0		6.6–5.3 6.0–5.8†	6.4–6.3 6.4–6.8†	
Mil	6.7–1.8 6.7–1.9 7.8–1.7	7.9–4.0 7.7–5.3 7.8–5.2† 7.8–5.1†			
125 Iowa		6.2–6.0 6.5–5.5 7.9–7.4 7.8–7.1†			
11914	6.7–4.7				
Homologous relative titer‡ (homologous titer — heterologous titer) = heterologous relative titer.					
RO	8.0	7.4	6.4		8.5
Naj	4.3	8.0	7.3	8.1	
Mil	2.8	5.1	8.0		
125-Iowa		7.7		8.0	
11914	6.0				8.0
% relationships§					
RO	100				
Naj	68	100			
Mil	52	76	100		
125-Iowa		100		100	
11914	88				100

* Logarithm of the LD_{50} of virus neutralized by the homologous serum-logarithm of the LD_{50} of virus neutralized by the heterologous serum.

† Chicken sera, all other sera obtained from rabbits.

‡ The homologous titers rounded to 8; the highest whole number.

§ The cross reaction was determined by the application of the formula $R = \sqrt{r_1 \times r_2}$ to the logarithm of the titers and then expressed in %.

gous serum-virus combination prepared and tested at the same time.

Results. Significant differences were detected in 3 of 5 antiserum-virus reciprocal combinations (Table II). The differences (determined by the formula $R = \sqrt{r_1 \text{ x } r_2}$ of Archetti and Horsfall(6)) were expressed as 76, 68 and 52% relationship. A pair of viruses neutralizing in one direction and not in the other would be expressed as 50% relationship.

The RO antiserum, which detected greatest differences among a few strains was subsequently used in neutralization tests with 12 strains (Table III). The greatest serological diversity observed existed between the RO strain from California and 3 strains from the eastern hemisphere, Herts and Milano of Europe and Vict of Australia. One American strain, 125-Iowa, however, exhibited as much divergence from strain RO as did Herts. Serological differences may be of considerable importance as exemplified by the results obtained with RO antisera and Milano virus, *e.g.*, homologous combination, 7.8 embryo LD_{50} neutralized; heterologous combination, 1.7 embryo LD_{50} neutralized. In such instances the relationship of the pair would not be accepted by diagnostic laboratories as significant.

Differences between strains were frequently greater when one of the pair was used as the antigen than when the other was employed

TABLE III. Per Cent Relationship of 12 Strains of NDV as Determined by Use of RO Antiserum* in the Serum Neutralization Test.

Virus	Titer†	Difference	Relationship‡
RO	7.4–7.1	.3	96
Bl	7.1–6.6	.5	93
Roakin	6.7–5.1	1.6	76
Ixta	7.1–5.2	1.9	73
GB	7.1–5.2	1.9	73
11914	6.7–4.7	2.0	70
MD I	7.4–5.1	2.3	69
KM	7.4–4.4	3.0	60
Naj	6.7–3.7	3.0	55
125-Iowa	6.7–3.4	3.3	50
Herts	7.0–3.7	3.3	52
Vict	6.7–2.9	3.8	43
Milano	6.7–1.8	4.9	27
Virus and normal serum	6.7–1.5	5.2	22

* Prepared in rabbits and inactivated.

† Logarithm of the LD_{50} neutralized by homologous serum — logarithm of the LD_{50} neutralized by heterologous serum.

‡ $\frac{\text{Heterologous titer}}{\text{Homologous titer}} \times 100 =$ relationship.

(Table II). A few strains appeared to be similar in one cross: 11914 antisera with RO virus (6.4; 6.7) and quite different when the reciprocal cross was used, RO antisera with 11914 virus (6.7; 4.7). Sometimes the neutralization titer appeared to be greater using a heterologous serum than a homologous one (11914 antisera and RO virus) (6.4; 6.7). Strain 11914 with which this occurred most frequently has been found to protect chickens better against challenge with certain other strains than against itself(3). The sensitiveness of individual sera in detecting antigenic differences varied considerably, (Table II), but in general, chicken and rabbit antisera were equally sensitive in detecting differences among strains of NDV. The study of the antigenic diversity of strains of Newcastle disease viruses by the serum neutralization test while still in preliminary stages has revealed differences of diagnostic importance, and raises a question of the feasibility of using a single vaccine in all situations.

Summary. Seventeen heterologous combinations of Newcastle disease virus and antiserum have been used in serum neutralization tests. Of these combinations 13 exhibited a difference of greater than 1.0 log between the neutralization of the homologous and the heterologous virus by prepared antiserum. A difference of 5.0 logs was observed between 2 strains.

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Hemolysis of PR8-Virus Sensitized Erythrocytes.* (20755)

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Microbial components may be roughly separated into 2 general groups with respect to their ability to agglutinate erythrocytes of various species. One, consisting of various viruses and bacterial products, exhibits the well-known Hirst phenomenon or direct hemagglutination(1,2); and the other, consisting usually of polysaccharide components of various bacteria, sensitizes erythrocytes of various species to agglutination by antisera homologous to the adsorbed microbial components(3-5). In the case of the former group(6) and of the latter group(5,7-10) it has been found by several workers that following the addition of antiserum homologous with the adsorbed microbial component, the addition of complement (C′) brings about lysis. This type of hemolytic system, therefore, differs from the usual complement-fixation system in that the antibody is directed not toward the erythrocyte but to the adsorbed component.

One phase of a related problem in which we are interested directed our attention to a study of the conditions under which the influenza virus could be involved in the lysis of erythrocytes. A survey of the literature available to us failed to reveal any reports upon the lysis of influenza virus-sensitized erythrocytes under the influence of C′, in spite of the generally accepted procedure of inactivating sera before use in the hemagglutination-inhibition test(11). This report will describe some results obtained from such a study.

Methods. Veronal buffer, pH 7.4(12) was

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used as diluent in all hemolytic procedures, and 0.85% NaCl was used in all hemagglutination (HA) tests. Erythrocytes were collected from several individuals of the same species in Alsever's solution and stored as such at 4°C. For use, erythrocytes were washed 3 times in the appropriate diluent and pooled from several individuals. Sharp and Dohme Lyo-complement was used as the source of guinea pig C′ and C′ from other species was collected and stored as serum at —20°C and used within a few days. The egg-adapted departmental strain of the PR8 influenza virus was used throughout this study, representing a pool of chorio-allantoic fluid (CAF) virus possessing an original hemagglutinating titer of 1:2,560(11). HA and hemaggluation-inhibition (HAI) tests were performed as described by the Expert Committee on Influenza(11), except that a 0.25% suspension of erythrocytes was used. Antiviral serum was prepared in rabbits and used as a pool of sera collected from several immunized rabbits and stored at —20°C. Normal rabbit serum was prepared in a similar fashion from non-immunized rabbits. All sera and C′ were subjected to multiple adsorptions with appropriate erythrocytes sufficient to remove naturally occurring antibodies before use in tests. Hemolytic titrations generally followed the procedure of Kabat and Mayer(12) and Mayer *et al.*(13). K values (50% hemolytic end-points of C′) were determined by plotting curves on log-log paper, following the equation of von Krogh (12), and taking the x-values where values of $y/1 - y = 1.0$. 1/n values, or the slopes, were also measured from the curves. y values (% hemolysis) were calculated from optical density (O.D.) determinations, using a Coleman Universal spectrophotometer at 550 mμ, which wavelength was selected on the basis of spectral-transmittance curves of hemoglobin from chicken erythrocytes.

Results. In a preliminary study it was found that guinea pig C′ did not lyse several species of erythrocytes, including those of the chicken, which had been sensitized only with virus. On the other hand it was found that chicken erythrocytes which had been sensitized with virus at 4°C and washed, and in the presence of anti-PR8 rabbit serum were lysed by guinea pig C′. Such findings resemble those of Fisher and Keogh(6) who worked with a hemagglutinin from *Hemophilus pertussis.*

In further studies it was found that C′ from the guinea pig, dog, rabbit, turkey, chicken, duck, and human were all active in lysing PR8-sensitized chicken erythrocytes in the presence of anti-PR8 rabbit serum. All of the various species of C′, as well as the anti-PR8 rabbit serum, were previously adsorbed with chicken erythrocytes, and the human C′ required additional adsorption with centrifuged PR8 virus because it was found to contain PR8 antibodies.

Preliminary studies of optimal conditions for this hemolytic procedure led us to accept the following constant conditions, some of which would possibly vary with different pools of virus and antivirus: One volume of washed, packed chicken erythrocytes was sensitized at 4°C for 90 minutes with 100 volumes of virus diluted 1:10. The sensitized erythrocytes were then washed twice at 4°C and resuspended in veronal buffer to a final concentration of approximately 1.4%. The density of this suspension was further adjusted so that 1.0 ml, when completely hemolyzed with 6.0 ml distilled water, gave an O.D. of 0.50 ± 0.2. Equal volumes of this sensitized suspension and the appropriate antivirus dilution were thoroughly mixed and allowed to stand for 10 minutes at room temperature. Two ml portions of this mixture were pipetted into a series of tubes, to which varying volumes of a constant C′ dilution were pipetted and the total volume in each tube brought to 7.0 ml with veronal. The tubes were stoppered, incubated at 37°C in a water bath for one hour with mixing at 10-minute intervals, centrifuged, and the O.D. values read on the supernatants. Plotted points were averages of duplicate tube readings. Other conditions of this procedure, including controls, have been discussed at length elsewhere(12,13).

Fig. 1 shows the results of a typical titration. It can be seen that K values generally increase with an increase in the dilution of antivirus. The exception is the 1:10 dilution

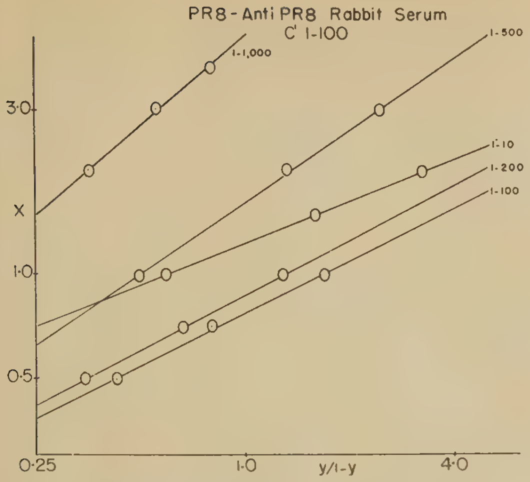

FIG. 1. Hemolysis of PR8 virus-sensitized chicken erythrocytes in the presence of variable antivirus dilutions (1:10 to 1:1000) and C′ volumes. Log-log scale. x equals ml of C′ and y the % hemolysis in the von Krogh equation: $x = K\left(\frac{y}{1-y}\right)^{1/n}$, where K is the 50% hemolytic unit of C′ and 1/n is the slope of the curves.

of antivirus which shows an inhibition of C′ activity resembling the Neisser-Wechsburg phenomenon. More interesting is the increase in slopes of the curves with increase in antivirus dilution. It is apparent that the 1:1,000 dilution of antivirus contains insufficient antibody to sensitize over some 50% of the cells to hemolysis by volumes of C′ permissible in our procedure. One might expect this curve to increase sharply beyond this last point, which finding has been substantiated in other experiments. In order to be able to plot points on both sides of the point of 50% hemolysis (y/1 — y = 1.0), antivirus dilutions of 1:500 or less were used thereafter.

The increase in slope with corresponding increase in antivirus dilution noted in Fig. 1 and Table I is not typical of hemolytic curves involving chicken erythrocytes sensitized with anti-chicken erythrocyte rabbit serum (Table II). It may be seen in Table II that the slopes are quite constant over antibody (hemolysin) dilutions from 1:10 to 1:1,000.

Control experiments were set up, following exactly the same titration procedure described above, except that: a) Pooled CAF from non-infected embryos was used to treat the erythrocytes in place of CAF virus. Such treated red cells were then mixed in separate titrations with both anti-PR8 rabbit serum and pooled serum from non-immunized rabbits, and b) virus sensitized erythrocytes were titrated with pooled serum from non-immunized rabbits. No hemolysis occurred in either a) or b).

Although it is tempting to speculate upon the increasing 1/n values as being a reflection of the combination of virus and antivirus in variable proportions, the possibility of interaction between some normal CAF component and anti-PR8 rabbit serum influencing the slopes was considered. Therefore, a titration identical with that carried out for Fig. 1 and Table I was performed, with the exception that antivirus was previously adsorbed with a sedimentable component from normal pooled CAF. Approximately 18 ml anti-PR8 rabbit serum (diluted 1:5) was adsorbed with sediment from 76 ml pooled normal CAF obtained by centrifugation at 24,000 G at 4°C for one hour. Such adsorption did not appreciably alter the change in 1/n values.

Since both the sedimentable and more soluble components of CAF influenza virus are known to fix C′ with their respective antibodies, the nature of the component responsible for this hemolytic reaction was studied.

TABLE I. K and 1/n Values of Hemolytic Curves of Virus-Antivirus Sensitized Chicken RBC. C′ dilution 1:25.

Antivirus dilution	K (ml)	1/n
1:10	.33	.36
1:100	.21	.58
1:200	.26	.62
1:500	.57	.85
1:1000	1.52	.87

TABLE II. K and 1/n Values of Hemolytic Curves of Hemolysin-Sensitized Chicken RBC. C′ dilution 1:50.

Hemolysin dilution	K (ml)	1/n
1:2	.26	.15
1:10	.19	.28
1:100	.47	.27
1:500	.62	.27
1:1000	1.02	.28
1:5000	2.50	.78

TABLE III. Activity of Virus Components Separated by Centrifugation.*

		Hemolytic procedure		
		100% C′ titration,	50% C′ titration	
CAF virus	HA titer	unit of C′	K (ml)	1/n
Before centrifugation	1:200	.05†	.96	.62
Supernatant	1:400	>.20	—‡	—
Sediment	1:10	.05	.48	.33

* CAF virus centrifuged at 24500 G at 4°C for 90 min.

† ml C′ (1-5) required for 100% lysis.

‡ Insufficient hemolysis with C′ volumes used to enable plotting significant curve.

TABLE IV. K and 1/n Values of Hemolytic Curves of Inactivated Virus-Antivirus Sensitized Chicken RBC. C′ dilution 1:25.

Antivirus dilution	K (ml)	1/n
1:10	.66	.38
1:100	.36	.55
1:200	.65	.66

Pooled PR8 virus was centrifuged at 24,500 G at 4°C for 90 minutes. The upper half of the supernatant and the sediment resuspended in the bottom half of the supernatant fluid were both studied for hemagglutinating activity and activity in the hemolytic reaction. In the hemolytic titrations, both 50% and 100% hemolysis end-points of C′ were determined. 50% end-points were determined as described above and 100% end-points were determined following the same general protocol, except that 0.2 ml volumes of 1.0% suspension of sensitized erythrocytes were mixed with 0.2 ml volumes of antivirus (diluted 1:100), varying C′ volumes from 0.01 to 0.2 ml were added, and total volumes brought up to 1.0 ml with veronal buffer. Table III shows that activity of the virus in this hemolytic reaction follows that of the hemagglutinating particle.

The possibility that elution of virus, under our conditions of incubation at 37°C, might be responsible for the change in slope noted in Fig. 1 was investigated. The same procedure for hemolytic titrations was followed with the exception that virus, which had been inactivated at 56°C for 30 minutes, was used. This inactivation was found to reduce the HA titer only slightly but to markedly inhibit elution at 37°C. Table IV shows that such inactivation did not appreciably alter the change in slopes from those with non-inactivated virus (Table I). This might indicate that the lytic process had begun soon after the C′ was added at 4°C or before much virus had eluted at 37°C.

Summary. A hemolytic system is described, which involves the lysis of virus-antivirus sensitized chicken erythrocytes by C′ and apparently depends upon specific interaction of virus and antivirus. Hemolytic curves resemble the usual erythrocyte-hemolysin hemolytic curves except that the slopes of the curves in the former increase upon dilution of antivirus. Activity of CAF virus in such a system apparently follows that of the hemagglutinating particle. Inactivation of the virus at 56°C for 30 minutes, which markedly inhibited elution at 37°C, had no appreciable effect upon such hemolysis by C′ in terms of the change in slopes. Changes in 1/n values were also not appreciably altered by previous adsorption of antivirus with a sedimentable component from pooled normal CAF.

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Poliomyelitis Virus Antibody in Different Lots of Human Serum Gamma Globulin.* (20756)

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The use of human serum gamma globulin in studies on the prophylaxis of poliomyelitis(1, 2) has created an interest in the antibody content of different samples of this substance. Published reports dealing with the quantitative determination of poliomyelitis antibody in gamma globulin preparations are those of Bodian(3) and Rhodes and Clark(4). Bodian, in tests on one lot of human serum gamma globulin employing a neutralization test in monkeys with 100 PD_{50} of virus, found antibody for all three immunologic types; 50% neutralizing titers of $10^{-2.0}$, $10^{-2.4}$, and $10^{-2.5}$ were reported for immunologic types 1, 2, and 3 respectively. Rhodes and Clark reported on the neutralizing substance demonstrable in mice for Type 2 poliomyelitis virus (Lansing); their studies included several samples of both placental and serum gamma globulins. Fifty-percent neutralizing titers of these preparations were about $10^{-3.5}$ using 8 to 85 LD_{50} doses in mice.

The present report deals with the antibody content of seven different lots of human serum gamma globulin tested in roller tube cultures of monkey kidney tissue; neutralizing activity against viruses representing the three immunologic types of poliomyelitis virus was determined.

Materials and methods. Gamma globulin. All human serum gamma globulin solutions used were from the supplies prepared for the American National Red Cross. Lot 116-1 represents the gamma globulin of serum collected from more than 20000 individuals by the American National Red Cross during World War II. This lot had previously been tested by Bodian(5) for Type 2 antibody in mice. The remainder of the preparations were kindly made available by Dr. W. McD. Hammon and had been used by him and his associates in their field trial studies(1,2). *Viruses employed.* The strains of virus used in these studies were Mahoney (Type 1), MEF-1 (Type 2), and Saukett (Type 3); the history of these strains and their adaptation to tissue culture have been described previously(6).

Neutralization tests. Preparation of cultures. Antibody determinations were carried out in roller tube cultures of cynomolgus monkey kidney tissue. Cultures were prepared with kidney fragments grown for 6 or 7 days under perforated cellophane in roller tubes; the medium employed for obtaining healthy epithelial outgrowth consisted of synthetic mixture 199(7,8) to which was added horse serum to a final concentration of 2% and penicillin and streptomycin in amounts of 100 units and 0.1 mg/ml respectively. The details of preparation of cultures and media will be presented in detail elsewhere. After 6 or 7 days of incubation, the medium used to obtain outgrowth of epithelial cells was removed and replaced by 1.5 ml of a 1:4 dilution of synthetic mixture 199 in Hanks' balanced salt solution (pH 7.4) to which was then added 0.5 ml of inoculum containing virus or virus-globulin mixtures. *Virus-globulin mixtures.* Serial 4-fold dilutions of gamma globulin were prepared using diluted mixture 199 (1:4) as diluent. To each dilution were added equal volumes of virus. Mixtures were incubated for one hour at room temperature before inoculation into cultures. The 0.5 ml inoculum for each culture contained 0.25 ml of virus dilution containing approximately thirty-two 50% tissue culture infecting doses (TCID_{50}) mixed with 0.25 ml of each dilution of gamma globulin. At least four cultures were used for each dilution. All samples of gamma globulin were tested simultaneously for antibody against each of the immunologic types of virus in order to minimize variations that may be attributable to testing on different occasions. Results were based on microscopic observations of cultures on the 3rd or 4th day following inoculation. All tubes showing typical signs of virus-induced degeneration were con-

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TABLE I. Neutralization of 3 Immunologic Types of Poliomyelitis Viruses by Different Lots of Human Serum Gamma Globulin.

Viruses (32 $TCID_{50}$)	Gamma globulin (lot #)	Gamma globulin dilutions[*] 64	256	1024	4096	16384	50% neut. end-point	Neg. log 50% neut. end-point
Type 1[†]	116–1		1/4[‖]	4/4	3/4	4/4	483	2.7
	157–3		0/4	2/4	4/4	4/4	1024	3.0
	169		0/4	3/4	4/4	4/4	634	2.8
	170		0/4	2/4	4/4	5/5	1024	3.0
	172		0/4	0/4	4/4	4/4	2048	3.2
	173		0/4	2/4	3/4	4/4	1392	3.1
	174		0/4	2/4	4/4	4/4	1024	3.0
							Avg	3.0
Type 2[‡]	116–1	0/4	0/4	4/4	4/4		512	2.7
	157–3	0/4	0/4	4/4	4/4		512	2.7
	169	0/4	1/4	4/4	4/4		404	2.6
	170	0/4	0/4	4/4	4/4		512	2.7
	172	0/0	0/4	3/4	4/4		634	2.8
	173	0/4	0/4	4/4	4/4		512	2.7
	174	0/4	0/4	4/4	4/4		512	2.7
							Avg	2.7
Type 3[§]	116–1	0/8	5/8	6/8	8/8		256	2.4
	157–3	0/8	2/8	6/8	8/8		512	2.7
	169	0/8	4/8	6/8	7/8		404	2.6
	170	0/8	1/8	8/8	8/8		458	2.6
	172	0/8	2/8	7/8	8/8		445	2.6
	173	0/8	3/8	8/8	8/8		338	2.5
	174	0/8	6/8	8/8	8/8		160	2.2
							Avg	2.5

[*] Expressed as reciprocal of the dilution.
[†] Mahoney Pool 34-SC7. Titer: $10^{-4.3}$.
[‡] MEF-1 " 22 . " $10^{-5.0}$.
[§] Saukett " 241-1 . " $10^{-4.5}$.
[‖] Numerator = No. of tubes showing cytopathogenic changes. Denominator = No. of tubes inoculated.

sidered infected. Titers were expressed as the dilution of serum added to the virus before inoculation into cultures and were calculated as the 50% neutralizing end points according to the method of Reed and Muench(9). A standardized hyperimmune serum and a sample of normal monkey serum were tested along with the gamma globulin samples; in addition, each test included a virus titration and uninoculated control cultures.

Results. The detailed results obtained are presented in Table I. It is apparent that all lots of gamma globulin tested contained antibody against the three immunologic types of poliomyelitis virus and in relatively uniform proportions. Titers of the different lots for Type 1 antibody were all within a range of 0.5 log; for Type 2 antibody the differences were within a range of 0.2 log; and, for Type 3 antibody, within 0.6 log. The arithmetic average of the logarithms of the titers of the seven lots for each of the immunologic types indicated that the dilution capable of protecting 50% of the cultures against Type 1 virus was $10^{-3.0}$, for Type 2 virus was $10^{-2.7}$, and for Type 3 virus was $10^{-2.5}$.

It is interesting to note that lot 116-1 was reported by Bodian in 1951 to give a neutralization end-point of $10^{-2.8}$ when tested in mice against thirty-two PD_{50} of Lansing virus. In the present study, tests performed in roller tube cultures of monkey kidney revealed that the titer of this pool was essentially the same ($10^{-2.7}$).

Assuming that the gamma globulin preparations employed represent about a twenty-three-fold concentration of the plasma content of this substance(3), it is possible to calculate the antibody content of the original plasma pools. Basing the calculation on the average neutralizing titers against the three types, it may be estimated that the average antibody values for the original plasma pools were $10^{-1.6}$ (1:44) against Type 1 virus, $10^{-1.3}$ (1:22) against Type 2 virus, and $10^{-1.1}$ (1:13) against Type 3 virus.

Summary. Neutralizing antibody content of 7 lots of human serum gamma globulin against the 3 immunologic types of poliomyelitis viruses were studied in roller tube cultures of monkey kidney tissue. Antibody against the 3 immunologic types of viruses was found to be present in all lots tested. The average of 50% neutralization end points in tests of the individual lots of gamma globulin for type 1 virus was $10^{-3.0}$, for Type 2 virus $10^{-2.7}$, and for Type 3 virus $10^{-2.5}$.

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Potentiating Effect of Iproniazid on the Pharmacological Action of Sympathomimetic Amines.* (20757)

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The antitubercular compound iproniazid (1-isonicotinyl-2-isopropyl hydrazine) was shown by Zeller *et al.*(1,2) to produce a marked *in vitro* and *in vivo* inhibition of mammalian monamine oxidase (MO) while isoniazid (isonicotinic acid hydrazide) was almost without effect. In accordance with these results Schayer demonstrated that iproniazid, but not isoniazid, reduces the deamination of C^{14} labeled epinephrine injected into rats(3). Since MO oxidizes and inactivates several other sympathomimetic amines such as phenylethylamine and tyramine(4,5,6) and thus may limit their pharmacological activity, the question arose as to whether or not the blocking of mammalian MO by iproniazid would change the response to these monamines. In this study the reaction of the nictitating membrane of the cat to the administration of various amines before and after the application of iproniazid is described.

Methods. Cats were anesthetized with pentobarbital and prepared for recording contractions of the right nictitating membrane and the carotid blood pressure. The injections of the drugs, except iproniazid and isonazid, were made into a saline infusion cannula and then flushed into the femoral vein with 2.5 to 3 ml of normal saline. Injections made in this way were prepared in 1 ml volume. Since iproniazid was found to cause equal enzyme inhibition whether given intravenously or intraperitoneally, 0.18 millimoles/kg were administered intraperitoneally. Isoniazid was administered in the same amount and manner. Phenylethylamine, tyramine and epinephrine were given in equal pressor doses to determine the contraction of the normal nictitating membrane; following this iproniazid was administered, and a period of 1 to 2 hours allowed to elapse to permit the enzyme to become inhibited. The three drugs were again administered in the same dosages as before. MO activity was determined for liver and brain homogenates ac-

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TABLE I. Cat Nictitating Membrane Responses to Intravenous Injections of Equal Pressor Doses of 3 Amines before and after Administration of 0.18 Millimoles/kg Iproniazid.

Injected amine	No. animals	Contraction index* Before iproniazid	Contraction index* 2 hr after iproniazid	T values
Phenylethylamine	6	.0 ± 0†	52.87 ± 19.18	2.75
Tyramine	5	.78 ± 1.09	76.46 ± 20.80	2.57
Epinephrine	8	3.71 ± .79	3.18 ± .62	.70

Control responses

Injected amine	No. animals	Contraction index Initial	Contraction index 2 hr after control, saline inj.	T values
Phenylethylamine	5	.32 ± .64	.09 ± .19	.14
Tyramine	3	3.59 ± 2.75	4.78 ± 2.23	.95
Epinephrine	7	3.30 ± .89	4.08 ± .93	.49

* Contraction index is avg value representing magnitude and duration of contraction of the nictitating membrane obtained by determining the area in square centimeters under the curve on the kymograph tracing by means of a planimeter.

† Stand. errors of the means are included.

The T values greater than 2 indicate the second response is statistically significantly greater than the first.

cording to the method described previously(1).

Results. The magnitude and duration of contraction of the nictitating membrane in response to the amines was found by circumscribing the perimeter of the curve with a planimeter and calculating the area under the curve in square centimeters. The mean area of response to similar doses of a given amine before iproniazid was found for a number of animals, and the mean for all animals tested was then determined. The responses 2 hours after iproniazid administration were determined in the same manner. The resulting values are indicated in Table I. These values may be compared with those determined on animals which were handled similarly except that iproniazid was not administered.

Two hours after the administration of iproniazid the response of the nictitating membrane to phenylethylamine rose from zero to a very marked contraction. Tyramine produces a measurable contraction of the nictitating membrane in normal animals in some cases, but this is greatly potentiated two hours after iproniazid administration. Fig. 1 shows a typical response of the nictitating membrane and blood pressure to tyramine before and after iproniazid. Epinephrine responses were essentially unchanged. Liver and brain homogenates were prepared for MO activity determinations from a number of the animals used in these experiments (Table II). Ammonia production and oxygen uptake drop sharply for both liver and brain, indicating a significant decrease in MO activity due to the administration of 0.18 millimoles/kg iproniazid.

Isoniazid used at the same molar concentration as iproniazid did not show a potentiating effect on the action of phenylethylamine or tyramine on the nictitating membrane.

Discussion. Iproniazid strongly potentiates the contraction of the nictitating membrane produced by phenylethylamine and tyramine. These results corroborate the experiments carried out with *in vivo* and *in vitro* studies on monoamine oxidase (MO), but they do not necessarily prove that the blocking of MO is the only or main reason for this observation. However, since MO is the only enzyme known which is inhibited much more by iproniazid than by isoniazid [isoniazid is more effective as an inhibitor of diamine oxidase(1) and spermine oxidase(7)] and since no potentiation takes place after the application of the latter drug, it is highly probable that MO is involved. The activity of MO appears to be a factor regulating the pharmacological action of phenylethylamine and tyramine.

No significant potentiation of the action of epinephrine on the nictitating membrane was noted. Again this does not necessarily mean that MO does not play some role in the metabolism of epinephrine as Schayer's work indicates. It is possible that epinephrine can

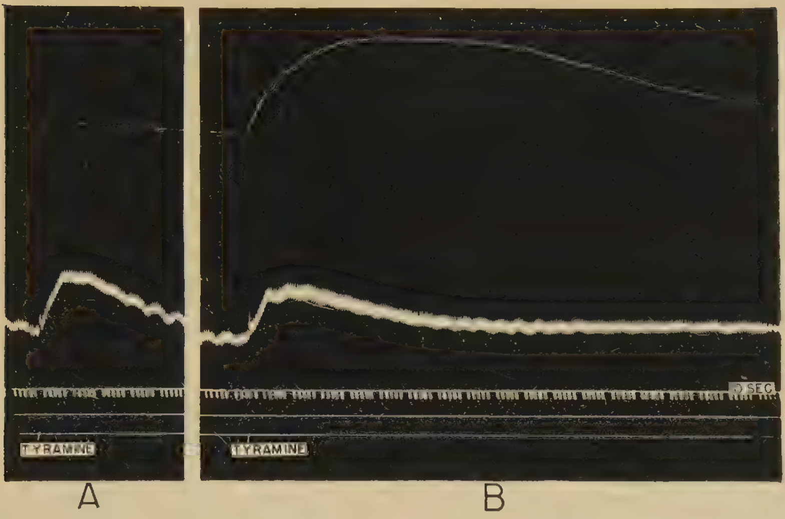

FIG. 1. Cat, 2.8 kg. Pentobarbital anesthesia. From top to bottom: Nictitating membrane; carotid blood pressure; time in 10 sec. intervals; zero blood pressure; signal. A. 5.7 × 10^{-3} millimoles (1 mg) tyramine hydrochloride intrav. B. Repeat dose of tyramine hydrochloride 2 hr after 0.18 millimoles/kg iproniazid.

utilize degradation pathways which are not available to the other two amines tested. Besides attack by MO epinephrine may undergo degradation by various other enzymes, attacking the catechol configuration and the alcoholic hydroxyl group. Iproniazid by inactivating MO blocks a greater part of the metabolic degradation of phenylethylamine than of epinephrine. The MO activity therefore, would be less of a limiting factor for the action of epinephrine than for phenylethylamine. Studies are being conducted on other related amines to determine whether or not their activity is influenced by MO inhibition. Experiments have indicated that tryptamine is potentiated while ephedrine and norepinephrine are not.

Blood pressure responses to phenylethylamine and tyramine were not potentiated by iproniazid. The exact explanation of this is not clear, but since a pronounced tachyphylaxis occurs with respect to the vasopressor responses, but not to the nictitating membrane responses, it is possible that the tachyphylaxis may obscure the potentiating effect of iproniazid.

TABLE II. Monoamine Oxidase Activity in Liver and Brain of the Cat after Administration of Iproniazid (0.18 Millimoles/kg).

	Normal values*	Iproniazid-treated animals
Liver	135.1 ± 2.88 (6)†	1.7 ± .5 (12)
Brain	17.8 ± 2.17 (5)	1.5 ± .4 (13)

* Expressed in terms of millimoles NH_3 produced per g of tissue in 2 hr incubation, employing 1 to 10 homogenates in M/15 phosphate buffer at pH 7.1, 1 ml/vessel with a final vol of 2 ml and a final concentration of tyramine · HCl of M/100. Stand. errors of the means included.

† No. of animals indicated in parentheses.

Summary. Iproniazid, in a dose of 0.18 millimoles/kg potentiates the action of phenylethylamine and tyramine upon the nictitating membrane of cats. The action of epinephrine is not changed significantly. Isoniazid does not have this effect when given in equivalent doses.

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Studies with Cysteinamine and Cysteine in X-irradiated Animals. (20758)

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Maisin *et al.*(1), have observed that post-irradiation cysteinamine (β-mercaptoethylamine) affords protection to rats if the liver has been shielded by lead. The role of the liver in this connection is not readily apparent. This phenomenon, however, is of considerable interest, particularly since one is prone to interpret pre-irradiation protection with chemicals of this sort in terms of the more or less immediate reactions of irradiated water. It has been shown recently, for example, that there is an inverse relationship between the protective efficiency of cysteine and ionization density which is comparable to that noted for the oxygen effect(2). Under the circumstances, it seemed worthwhile to attempt confirmation of the postirradiation action of cysteinamine and to include ancillary observations of the protective capacity and synergism of cysteinamine and cysteine.

Methods. 1) Cysteinamine and liver shielding in the rat. Male rats of the Holtzman and Sprague-Dawley strains weighing 190-225 g were used. The experimental procedure of Maisin *et al.*(3) was duplicated insofar as possible, except that the x-ray dose was 1000 r instead of 1130 r and that several dosages of cysteinamine were evaluated. The rats were divided into 4 groups. Group 1 received 1000 r total body radiation; Group 2 received 1000 r total body radiation plus cysteinamine* (17.5, 35 or 70 mg/kg, I.P.) immediately, 6 and 12 hours after irradiation; Group 3 had the hepatic region shielded by a 0.5 x 3.2 x 5.0 cm lead plate during irradiation; Group 4 combined liver shielding with the 3 postirradiation injections of cysteinamine. The lead shields were placed so as not to protect the splenic region. Radiation factors were 180 kv, 15 ma, 0.225 mm Cu HVL, 55 cm target distance, and 84 r/ min. dose rate. 2) Protective effects of cysteinamine and cysteine in mice. CF_1 female mice weighing 19-24 g were divided into groups of 25 animals each and irradiated in pairs with 1000 r. Immediately before irradiation, individual animals received intravenous injections of either 75 or 150 mg/kg of body weight of cysteinamine or 600 or 1200 mg/kg of cysteine. In other studies 75 mg/kg of cysteinamine and 600 mg/kg of cysteine were combined and injected immediately before irradiation. Radiation factors were 250 kv, 15 ma, 0.5 mm Cu and 3.0 mm Bakelite filters, 29.5 cm target distance and 200 r/min. dose rate. The supralethal dose of 1000 r (30 day LD_{95} for CF_1 mice = 800 r) facilitates comparison of the protective effect with minimal numbers of animals.

Results. About 60% of rats with the liver area shielded survived exposure to 1000 r, a dose that is lethal to over 95% of unshielded animals within 30 days. Postirradiation injections of cysteinamine alone did not afford any protection. Differences in lethality of irradiated animals treated with cysteinamine in combination with liver shielding and animals with shielding alone were not statistically significant (Table I). Thus, these experiments do not verify a postirradiation action of cysteinamine in liver shielded rats (1,3). The reason for this is not obvious. Animal strain, age, and weight were not specified in the report of a postirradiation effect (1,3). Dosage was stated as 7 mg per adult rat. Three dosages of cysteinamine were employed in the present experiments. These correspond to 7 mg per 100, 200, and 400 g rat and span the body weight range for adult animals generally. Subtle differences between animal strains may be contributory. It may be noted, however, that neither Holtzman nor Sprague-Dawley rats were affected by the treatment in question.

Turning to the protective effects of cysteinamine and cysteine when given prior to irradiation we may note that the former is more efficient than the latter. Maximally tolerated

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* Kindly provided by Dr. Harvey Blank, E. R. Squibb and Sons.

TABLE I. Effect of Cysteinamine on Survival of Rats Exposed to 1000 r X-rays with and without Liver Shielding.

Group	Dose (mg/kg)	Strain	No. of animals	% survival			
				1 wk	2 wk	3 wk	4 wk
Irradiated controls*	—	S.D. + H	66	23	4	4	4
Cysteinamine after irrad.†	17.5	S.D.	25	12	4	4	4
	35	H	41	32	7	7	7
	70	S.D.	24	17	4	4	4
Liver shielded*	—	S.D. + H	66	80	61	61	61
Idem.; cysteinamine after irrad.†	17.5	S.D.	22	91	73	73	68
	35	H	41	68	68	68	68
	70	S.D.	23	74	65	65	65

* Because survival patterns at 1000 r are similar in both Holtzman and Sprague-Dawley rats, controls are combined.

† Injected IP immediately after, 6 and 12 hr postirradiation.

doses, 150 mg/kg of cysteinamine and 1200 mg/kg of cysteine *I.V.*, afford comparable protection to mice against acute radiation lethality (Fig. 1). Cysteinamine is thus 5 times as efficient as cysteine on a molar basis. Since mice will not tolerate a combined injection of these dosages, 75 mg/kg of cysteinamine and 600 mg/kg of cysteine were employed in the additivity studies. Neither dose alone alters appreciably the absolute mortality resulting from exposure to 1000 r, although mean survival times are slightly prolonged. When given together, however, there is definite protection equivalent to that afforded by the full dosage of either substance alone.

The synergism between minimally effective doses of cysteinamine and cysteine may be taken as presumptive evidence that similar reactions are responsible for the protective effects. The greater efficiency of cysteinamine, when compared to cysteine, may be attributed to differences in the distribution and biological life of these substances. Cysteinamine, for example, may be oxidized in transit less rapidly than cysteine, which could account for its greater toxicity. The importance of the pharmacological attributes

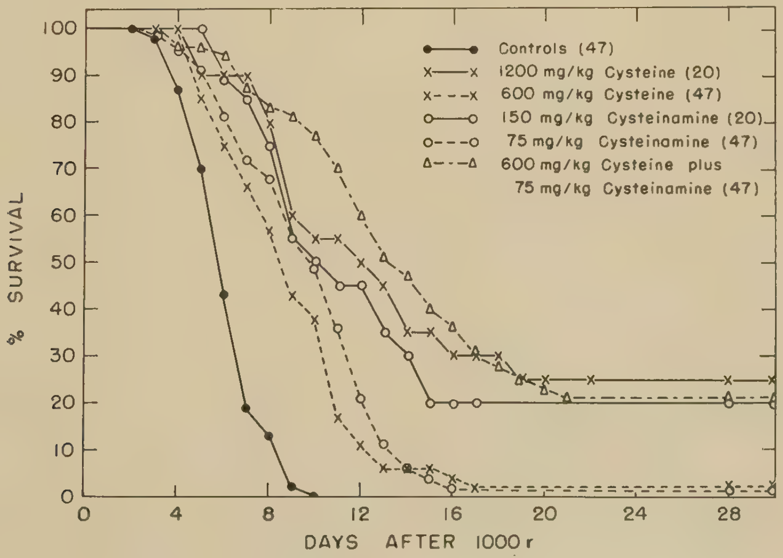

FIG. 1. Synergistic effect of cysteinamine and cysteine upon survival of mice following exposure to 1000 r total-body irradiation. Figures in parentheses indicate No. of animals in each group.

of the various substances is illustrated further by the fact that mercaptosuccinate is not protective, in contrast to cysteine, cysteinamine, glutathione and BAL(4). In perhaps the the same sense, it may be recalled that cystinamine, $(S{-}CH_2{-}CH_2{-}NH_2)_2$, is an effective protective agent under certain circumstances but that cystine is inactive. The effect of cystinamine, unlike cysteinamine or cysteine, is restricted to the intact animal. This suggests that an *in vivo* interconversion to an active form is necessary. Cystinamine, for example, does not modify the radiosensitivity of pea roots(7) or reticulocytes of dog's blood *in vitro*(8). Protection of the whole animal may, perhaps, be attributed to its reduction to cysteinamine(6) or to the rapid formation of histamine(7). Large doses of histamine and other amines have been shown to enhance the survival of irradiated mice perhaps because of altered tissue oxygen tension resulting from vascular effects(5,7). Cysteinamine, or the other hand, does not liberate histamine(7) and presumably exerts a more direct action as a consequence of its sulfhydryl group, an action comparable to that of cysteine. This is, in fact, suggested by the apparent additivity of the 2 compounds.

Summary. 1. The observation of a post-irradiation effect of cysteinamine in rats with the liver area shielded has not been verified. 2. Cysteinamine is about 5 times as effective as cysteine when given to mice intravenously in equimolar amounts prior to x irradiation. However, maximally tolerated doses of each give equivalent protection. 3. Cysteinamine and cysteine appear to be additive in their protective effect against acute radiation lethality. This is suggestive of a similar mechanism of action.

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Rapid Colorimetric Method for Determination of Isonicotinic Acid Hydrazide in Blood Plasma. (20759)

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(Introduced by B. J. Olson.)

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The observation that isonicotinic acid hydrazide (isoniazid*) and its isopropyl and glucose derivatives have activity against tuberculosis in experimentally infected laboratory animals and against human tuberculosis (1) has raised the need for methods of assay of these compounds in body fluids. Reports of 3 methods appeared simultaneously as follows: S. Rubin and his coworkers(2) described an oxidation procedure and assay of the resulting isonicotinic acid with cyanogen bromide and ammonia. The degree of sensitivity was not stated. B. Rubin and collaborators (3) employed a rapid extraction method, sensitive to 5 µg ± 10% per ml of blood plasma, which involved estimation of the ultraviolet absorption of an isoamyl alcohol extract. Kelly and Poet(4) extracted hydrazide from alkalinized plasma or urine by the addition of ammonium sulfate and an isoamyl alcohol-ether mixture, and after acidification, determined the ultraviolet absorption of the extract or the color with p-dimethylaminobenzaldehyde. The latter was reported to be sensitive to one μg ± 5% per ml of plasma.

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* See footnote 5, *Am. Rev. Tuberc.,* 1952, v65, 754.

The present report describes a procedure for colorimetric determination of isoniazid in blood plasma. The reaction results from condensation of isoniazid with glutaconic aldehyde formed by alkaline hydrolysis of 4-pyridylpyridinium dichloride. The intensity of the yellow color developed varies logarithmically with the concentration of isoniazid. This reagent has been described by Feigl(5) as useful in detecting small amounts of primary aromatic amines, but to our knowledge has not been used for measuring hydrazine and hydrazides.

Method. The determination is carried out as follows: Two ml of isoniazid solution containing 3% (w/v) aqueous trichloracetic acid are pipetted into a 5 ml volumetric flask. Five-tenths ml of 4-pyridylpyridinium dichloride reagent (1% aqueous solution of recrystallized Eastman product) is added, followed by 0.7 ml 2 normal sodium hydroxide, and 0.5 ml 2 normal hydrochloric acid. In the absence of trichloracetic acid, 0.5 ml of 2 normal sodium hydroxide is sufficient to liberate the glutaconic aldehyde. After adding distilled water up to volume and shaking, the mixture is held at room temperature for 15 minutes to assure maximum color development. The color is stable for at least an hour. The yellow solution is then transferred to a Fisher electrophotometer microcell and read against distilled water with the 425 mμ filter. A blank of the reagents and a standard solution containing 10 μg/ml of isoniazid are tested with each series of determinations.

Ten determinations were made of each of the concentrations such as might be found in body fluids, namely, 2, 4, 8, 12, 16, 20 μg. From the results in Table I, it is evident that the color intensity is proportional to the concentration of isoniazid, in accordance with Lambert-Beer's law, and that it is reproducible and sensitive. (2 μg per ml ± 3.2% in duplicate determinations).

Recovery from plasma. For *in vitro* recovery tests, varying amounts of isoniazid were added to normal guinea pig plasma containing ACD[†] as anticoagulant and to normal horse serum. Two ml of plasma or serum, diluted with 6 ml of distilled water, were mixed with 2 ml of 15% trichloracetic acid and held at room temperature for 15 minutes. After filtration, 2 ml portions of filtrate were measured into 5 ml flasks and the above procedure followed. Recovery of 10 μg of isoniazid added to one ml of guinea pig plasma was found to vary from 9.6 μg to 10.0 μg, 96 to 100%, and with one ml of normal horse serum averaged 9.5 μg or 95%.

TABLE I. Standardization and Reproducibility of Color Reaction of Isoniazid and Glutaconic Aldehyde from 4-Pyridylpyridinium Dichloride.

Isoniazid, μg	Optical density[*] Range of 10 determinations	Avg of 10 determinations	Sample avg minus blank avg
0	13.1-13.4	13.2	0
2	15.1-15.4	15.24	2.04
4	17.1-17.4	17.25	4.05
8	21.1-21.3	21.19	7.99
12	25.0-25.3	25.18	11.98
16	29.1-29.3	29.21	16.01
20	32.9-33.3	33.26	20.06

[*] Figures represent 100 × E/425 mμ, measured in Fisher electrophotometer 3 ml microcell.

Determination of isoniazid in blood plasma of animals under treatment. Each of 120 DBA mice, weighing 20 to 25 g, was given an oral dose of 5 mg of isoniazid. Then in groups of 15 the animals were bled ½, 1, 1½, 2, 3, 4, 7, and 18 hours after the drug administration. Using ACD as anticoagulant, the bloods were pooled from each group of 15 mice and the plasma separated. Blood plasma from 15 untreated mice served as control. Duplicate portions of the trichloracetic acid filtrates from the plasma were assayed for isoniazid. The readings were corrected for the slight amount of chromogen found in the untreated mouse plasma. The averages of results from 2 series of experiments were plotted against corresponding times as shown in Chart 1. The curve reveals that the maximum plasma peak of 6.5 mg per 100 ml was reached in ½ hour, with a sharp decline in 4 hours, and a negligible amount or no drug at all in the 18-hour

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[†] ACD contains 8 g citric acid, 22 g sodium citrate, 24 g dextrose in 1000 ml water; 0.5 ml solution is added to 5 ml blood.

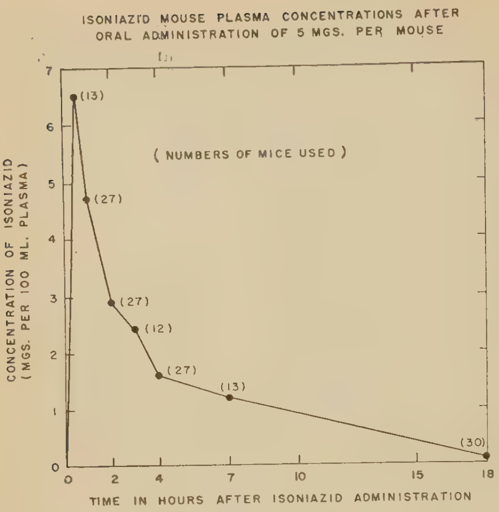

CHART 1

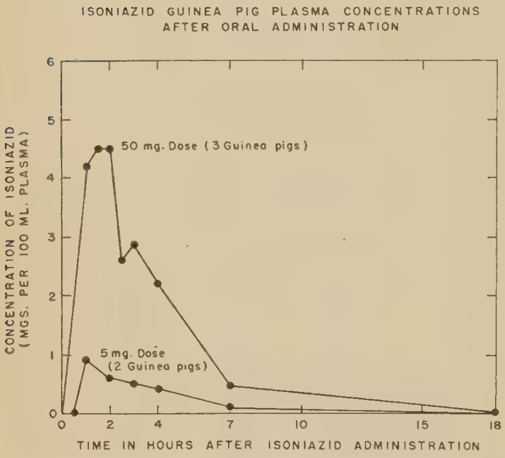

CHART 2

sample. Determinations of one-hour plasma levels were repeated in 2 additional experiments with results of 5.1 and 4.7 mg per 100 ml. These results approximate the average figure of 4.4 mg per 100 ml reported by Rubin (3) as present one-half hour after administration of the drug in mice.

Guinea pig plasma levels were determined in the same manner on the pooled plasma from 2 animals, each of which had received a 5 mg oral dose of isoniazid, and also on plasma from 3 animals after a 50 mg oral dose. The results are plotted in the curves in Chart 2. As expected, higher levels were obtained after the 50 mg dose than were found after the 5 mg dose. In both instances the maximum concentration was reached within 2 hours after administration of the oral dose of isoniazid.

Discussion. Although other concentrations of reagent, and of acid, alkali, and protein precipitant have been tested, the optimum concentrations found for reproducibility in this test differ but slightly from those reported by Feigl for his aromatic amine spot test. Hence plasma levels of isoniazid may be increased by the presence of such aromatic amines as the sulfonamide drugs or p-aminosalicylic acid. Since normal animal plasma have shown small amounts of other chromogens, a control determination on the plasma before isoniazid administration is essential.

Summary. The color reaction between isonicotinic acid hydrazide (isoniazid) and glutaconic aldehyde liberated from 4-pyridylpyridinium dichloride as described conforms to Lambert-Beer's law and is the basis for a sensitive and rapid method for determination of hydrazides in blood plasma. Blood plasma levels in mice and guinea pigs after oral administration of respectively 5 and 50 mg of isoniazid have been found to reach a maximum within 30 minutes to 2 hours and to fall to low levels within 18 hours.

We wish to acknowledge the interest and helpful suggestions of Dr. W. C. Hess, Professor of Biochemistry at Georgetown University School of Medicine.

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Investigation of Serum Protein Patterns in Patients Undergoing Operation. (20760)

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The disturbance of protein equilibrium following injury has been extensively studied in humans as well as in experimental animals(1,2). A negative nitrogen balance occurring as the first phase of the changed metabolism, and designated by Browne *et al.* (3) as the catabolic phase, is followed during the convalescence by an anabolic phase characterized by a positive nitrogen balance. The changes in the serum protein pattern of 6 patients following injury or surgery has been investigated by Cuthbertson and Tompsett (4). They found a decreased albumin and increased globulin fraction following injury. The serum components were separated by neutral salt precipitation. By this method the α_1 and α_2 globulins are not separated from the albumin.

The recent development of paper strip electrophoresis(5) made possible the study of the changes of all protein components in small amounts of blood without causing any discomfort to the patients. The result of such an investigation on patients undergoing surgery is presented.

Procedures and method. The sera of 45 patients were investigated. The patients were grouped in 3 main categories: patients with tuberculosis undergoing chest surgery, patients with uro-genital trouble, mostly benign prostatic hypertrophy, and patients with gastrointestinal and pulmonary cancer. Both sexes of the white and Negro races were represented. The ages of the patients were between 17 and 84 years. Venous blood was collected 8 to 12 hours before operation, then at intervals 4 to 96 hours after operation, and up to 12 days when the patient was available. On each serum the total protein concentration was determined by a modified Linderstrøm-Lang specific gravity gradient(6). On most of the patients the hematocrit was determined pre- and post-operatively by the Wintrobe method. The separation of the protein components was carried out by paper strip electrophoresis in a tent type Durrum apparatus at room temperature. Strips of Whatman 3 MM filter paper 2.5 cm x 38 cm were used with veronal buffer of pH 8.55 and μ 0.05. A current of 2-2.5 ma per strip was applied for 7 hours at which time the albumin had migrated 8-10 cm. The strips were dried for 10 minutes at 110°, stained with bromophenol blue (0.01% bromophenol blue, 5% acetic acid, and 5% mercuric chloride) for 6-12 hours, washed 3 times with 2% acetic acid 10 minutes each time, and once with 2% acetic acid containing 0.5% sodium acetate. They were then blotted and dried for 10 minutes at 110° and cut at positions of least staining between the bands. The color was eluted from the finely cut paper fragments with 6 ml of 0.01 N NaOH and the optical density read in a Coleman Universal Spectrophotometer at 590 mμ.

Experiments were carried out to establish the effect of the time of drying, of washing, and of staining on the reproducibility of color readings. The drying of the strips between 10 and 30 minutes at 110°, the washing of the strips 10 minutes to 24 hours, and the staining between 6 and 48 hours had no significant effect on the estimated proportions of the components. The elution of the color was complete in about 1½ hours, after this time the color values found were constant up to 24 hours. If the strips, however, were stored, stained and dried but not eluted, the color faded measurably after 4 days. The fading of the albumin was more rapid than that of globulins, which resulted in too low albumin values. By repeated estimations of serum patterns of different samples from the same individual, differences of ±4% in the albumin, and differences of ±2% in the other serum components was found statistically sig-

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* Supported in part by a Massengill Fellowship.

TABLE I. Serum Protein Patterns of Clinically Healthy Persons and Patients Prior to Operation.

Diagnosis	No. of cases	Total protein, g/100 ml Mean	Avg	Albumin* Mean	Avg	Alpha$_1$* Mean	Avg	Alpha$_2$* Mean	Avg	Beta* Mean	Avg	Gamma* Mean	Avg
Healthy	20	8.1	7.35	68.0	58.7	8.6	5.9	16.2	9.5	18.2	10.7	21.6	14.4
		7.1		47.8		4.1		4.1		7.5		10.4	
T.B.	24	8.3	7.0	65.6	54.5	7.0	5.9	15.5	11.0	15.8	11.7	24.4	17.4
		6.4		41.5		3.9		8.1		6.4		9.9	
Ca	7	9.9	7.8	53.5	46.7	8.1	6.8	16.1	13.3	18.7	13.5	31.6	18.4
		6.2		31.8		5.4		11.3		11.1		9.4	
Bg. prostatic obst.	8	8.5	6.8	53.2	48.6	9.0	7.7	19.8	14.1	15.7	13.1	19.9	16.9
		6.9		40.0		5.9		9.3		11.4		12.9	
Misc.	6	8.4	7.1	58.0	48.0	8.4	6.1	13.8	10.8	13.4	12.8	33.7	22.0
		6.9		31.9		4.6		8.3		11.8		16.2	

* Values are % of total protein.

TABLE II. Serum Protein Patterns in Patients Showing High Gamma Globulin.

Diagnosis	Total protein, g/100 ml	Albumin*	Alpha$_1$*	Alpha$_2$*	Beta*	Gamma*
Ca of kidney	7.6	44.7	4.8	9.6	13.4	27.5
Bronchiectasis	8.4	31.9	7.2	13.8	13.3	33.8
Pulmonary T.B.	7.7	47.8	3.9	8.5	9.7	30.1
Pulmonary Ca	8.1	31.8	8.1	15.1	12.7	31.3
Empyema	7.4	40.0	7.0	11.0	10.0	32.0
Pulmonary T.B.	8.7	38.5	6.2	10.5	10.0	34.8

* Values are % of total protein.

nificant ($P > 0.05$). In evaluating the post-operative sera these figures were taken as a basis for designating changes.

Results. No differences were found in the serum proteins with regard to the sex, race, or age of the patient. The type of narcosis used did not seem to influence the serum protein pattern. The preoperative sera of patients were compared with sera from clinically healthy persons (Table I). The total protein concentration was about the same in all groups. In the preoperative groups the albumin tended to be lower and the globulins higher than in the healthy controls. This difference was least pronounced in cases of patients with tuberculosis. All these patients were treated preoperatively for a prolonged time in tuberculosis sanatoriums where they received a high protein high caloric diet. The mobilities of the components in the pre-operative sera did not differ from the healthy persons except in 3 cases in which an additional α_2 (2 cases) and an additional beta globulin (one case) was found. Increased gamma globulin, higher than 25% was found in 6 cases (Table II). Five of these had advanced pulmonary disease.

The protein patterns in healthy persons did not vary significantly over many days (Table III). Changes found in the serum patterns of patients undergoing surgery are shown in Fig. 1 and Table IV. Immediately after operation the albumin dropped in one-third of the cases, and the α_1 and α_2 globulins

TABLE III. Variations in Composition of Serum of Clinically Healthy Persons.

	Total protein, g/100 ml	Albumin*	Alpha$_1$*	Alpha$_2$*	Beta*	Gamma*
1	7.1	67.1	4.7	7.4	9.5	11.3
	7.9	64.4	5.9	9.1	10.0	11.6
	7.1	67.5	6.0	7.6	8.1	10.8
2	7.1	59.1	6.4	10.5	10.9	13.1
	7.1	60.0	5.8	11.1	12.6	10.5
	7.1	56.5	5.9	12.4	12.8	12.4
3	7.3	61.5	5.4	9.2	8.6	15.3
	7.5	59.5	6.1	8.8	8.6	17.0
	7.5	64.2	5.0	9.0	8.3	13.5
	7.3	63.0	5.7	8.5	9.1	13.7
	7.5	65.0	4.4	6.6	8.5	15.5
	7.7	62.5	5.0	6.5	10.0	16.0
	7.5	64.2	3.7	7.8	9.1	15.2
4	7.6	47.8	6.9	10.5	11.9	22.9
	7.5	49.0	6.3	10.6	12.4	21.7
	6.1	51.0	6.0	10.1	11.5	21.4

* Values are % of total protein.

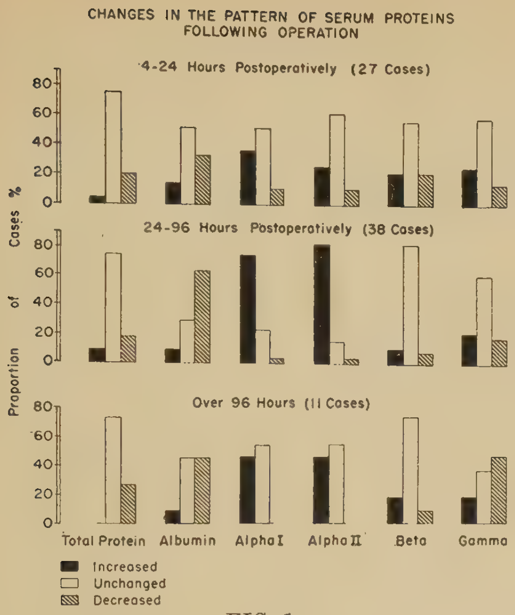

FIG. 1

rose. In the following 3 days in 68% of all cases the albumin was significantly decreased, the α_1 and α_2 globulins were increased in 76% and 82% of the cases, respectively. The beta globulins remained unchanged in 82% and the gamma globulin in 61% of all cases. The mean of the total proteins did not change more than by 1 g/100 ml during the time of observation. The hematocrit in the preoperative patients was generally slightly below normal (mean 42% for both sexes), it increased slightly in the first 24 hours postoperatively (mean 45%), and it decreased during the following 4 days (mean 40.8%). No significant difference was found in the post-operative response in the groups of patients.

The components of serum proteins did not show differences in their mobilities before and after operation with the exception of 3 cases in which the samples taken after operation showed components of different mobilities. As all the patients received blood, saline, or glucose in quantities of 500 to 2500 ml during and immediately after the operation, the absence of changes in the total proteins or protein patterns was remarkable.

Discussion. When evaluating the changes in the serum protein patterns following surgical operations it should be borne in mind that the preoperative levels of the components in this series and presumably in all other similar investigations have already shifted in a direction of the levels found in the postoperative period. The decreased albumin together with an increased globulin level prior to operation might be ascribed to protein depletion of the patients due to their respective diseases. It has been observed that a negative nitrogen balance does not occur after injury in patients who have very little nitrogen reserve due to undernourishment or protein depletion. The gradual decrease of the serum albumin in the first postoperative days is in agreement with the findings of Cuthbertson(7) and of Grossman *et al.*(8) that the nitrogen excretion in urine was not great immediately after operation and that only after the lapse of 3 to 4 days did it become considerable.

TABLE IV. Changes in Serum Albumin, $Alpha_1$, and $Alpha_2$ Globulins Following Surgery.

Time	No. of cases	Albumin* Range	Albumin* Mean	$Alpha_1$* Range	$Alpha_1$* Mean	$Alpha_2$* Range	$Alpha_2$* Mean
Pre-op.	45	68.0 47.8	51.2	8.6 4.1	6.3	16.2 4.1	11.4
Post-op.							
4-24 hr	27	55.1 45.0	46.9 (9)†	14.0 7.9	9.1 (10)	21.0 12.4	14.5 (7)
24-96 "	38	50.8 21.8	41.5 (24)	12.6 8.1	9.6 (28)	19.2 12.3	15.1 (31)
96+ "	11	49.0 38.4	44.8 (5)	10.7 7.4	9.9 (5)	20.0 16.0	16.4 (5)

* Values are % of total proteins.

† Figures in parentheses represent No. of sera in these groups in which changes were statistically significant, *i.e.*, greater than 4 for albumin or 2 for the globulins.

The mechanism and biological significance of the negative nitrogen balance after injury is not clear. It is generally assumed that the direct destruction of tissue during operation is not the responsible factor. Hormonal and enzymatic factors might be decisive. There are many similarities in the nitrogen metabolism in response to injury and the general "alarm" or "adaptation" syndrome(9). The adrenocortical hormone of the pituitary(10) and the 11-oxycorticoid(11) produced negative nitrogen balance. Apart from the decrease of the total mass of liver proteins, the concentration of protein enzymes(12,13) including D-amino acid oxidase(14) was found to be decreased in the liver of rats fed a low protein diet. A disturbance of the enzyme systems of the liver, especially those connected with the protein metabolism, may be at least in part responsible for the postoperative protein disequilibrium.

Summary. 1. Distribution of serum protein components was investigated by the use of paper strip electrophoresis in 45 patients undergoing surgery. 2. A significant decrease in the albumin and increase of a_1 and a_2 globulins occurred in one-third of the cases during the first 24 hours following operation. These changes were present in 80% of all cases during the following 4 days. Thereafter the serum components tended to return to their preoperative levels. Components with mobilities different from those found preoperatively occurred in 3 cases after operation. 3. Total serum proteins and the hematocrits did not show any significant variation in the early postoperative period.

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An Electrophoretic Method for Titration of Antisera and Its Application to Anti-Tumor Sera.* (20761)

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Coulter(1) reported that the electrophoretic mobility of washed sheep erythrocytes which had been sensitized with homologous antiserum was less than that of normal washed cells. Shortly thereafter De Kruif and Northrop(2) concluded from their observations on the behavior of *Bacillus typhosus* in the presence of its homologous antiserum that the antibody combines with the surface of the bacteria, producing a surface charge density dependent upon the proportion of the surface so combined. This specific effect of homologous antiserum has since been demonstrated for the

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erythrocytes of several species and for numerous species of bacteria. Shibley(3) further showed for a variety of bacterial species that the electrophoretic mobility continued to change with increasing concentration of antiserum with an apparent convergence upon a common value, shown to be that of denatured pseudoglobulin.

From consideration of these early data it appeared probable that an electrophoretic technic could be employed for the titration of antisera with respect to their combination with the cell surface, as distinct from any secondary reaction possibly following combination. Such a method should contribute to the fundamental study of antigen-antibody reactions as well as provide a practical method for the assay of antisera in which either no secondary reaction occurs or in which such a reaction cannot be suitably measured. The work presented here describes the development of such a method using cells of the Gardner Mouse lymphosarcoma (6-C3H-ED) and homologous antiserum for which no suitable *in vitro* assay method had been developed. Our objective has been the development of the simplest and most rapid method that would give a precision equivalent to that of a doubling dilution tube serological test.

Methods and materials. Tumor: The Gardner lymphosarcoma 6-C3H-ED was grown as an ascites tumor. The line was started by the intraperitoneal injection of 0.5 ml of 20% whole cell homogenate, and thereafter line transfers were made at seven-day intervals by the intraperitoneal injection of 0.5 ml of ascitic fluid. *Buffer:* The buffer used was M/15 sodium potassium phosphate buffer, pH 7.3, diluted with 9 vol. of a 5.4% glucose solution. The capacity of this buffer was sufficient to maintain the pH of the sample at pH 7.3 ± 0.2 throughout all the necessary manipulations. The advantages of this buffer in electrophoresis have been described elsewhere(4). *Antisera:* Antisera were prepared by the immunization of rabbits with either whole tumor cells from ascitic fluid (No. 4, No. 5b, and No. 6) or with a homogenate of lymphosarcoma tissue (No. 9 and No. 10). *Preparation of the samples for electrophoresis:* The ascitic fluid was centrifuged for 15 minutes at 2800 rpm in a small angle head machine. The resulting pellet was twice washed with buffer and was then made up to a concentration by volume of 0.2%. The pellet was uniformly suspended by forcing the suspension through a 21-gauge syringe needle. Approximately 90% of the cell concentration appeared as single cells. The concentration of erythrocytes was very low. Dilutions of antiserums were made by the addition of the required amount of serum to the buffered cell suspension.

It is obvious that variation among samples standardized in this manner will be high. However, for all cell concentrations suitable for microelectrophoresis (approximately 0.1% to 0.4%) the mobility was found to be independent of the cell concentration. Measurements of the electrophoretic mobility of tumor cells made at periods from 0-20 hours after the addition of antiserum failed to reveal any significant change with time. Therefore, the measurements were made 15-30 minutes after the addition of antiserum.

Electrophoresis apparatus and technics: The electrophoresis apparatus was that described by Hartman, Bateman, and Lauffer(4) with the cell oriented in the lateral position. This orientation, in combination with an eyepiece reticle having a rectangular grid, allows the measurement of the horizontal component of travel (the elecetrophoretic velocity) while the cells are sedimenting through a distance up to 500 μa. At currents from 0.25-0.50 ma, satisfactory data were obtained at all concentrations of antiserum as long as the tumor cell concentration was held within the range recommended above. The excursion times of 10 individual cells were measured, the electric field then reversed, and a similar set of measurements were made on 10 different cells. Measurements were made at the upper stationary level only.

Neutralization test: Antisera were doubly diluted from 1:2 to 1:64. To the serum dilution was added an equal volume of a 2% homogenate of lymphosarcoma tissue in saline. Normal C3H mice were then inoculated subcutaneously with 0.2 ml of the tumor-antiserum mixture in both the right and left groin regions.

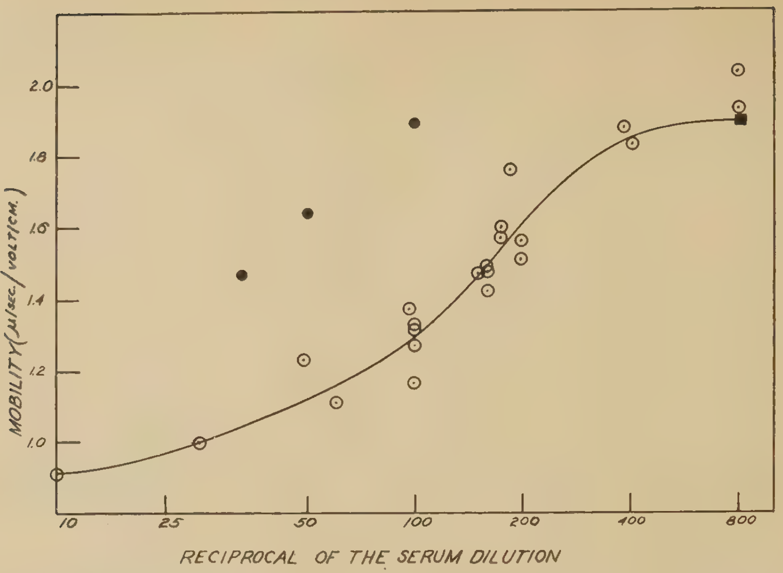

FIG. 1. Electrophoretic mobility at 25°C of lymphosarcoma cells in the presence of antiserum diluted with M/150 phosphate buffer containing 4.85% glucose, pH 7.3. All migration was toward the anode. ⊙ homologous antiserum. ● normal rabbit serum. ■ avg value in buffer only.

Discussion. It is obvious that, given an antiserum of sufficient antibody content, the cell mobility versus antiserum dilution curve will, at high dilution, approach asymptotically the mobility in buffer alone and, at low dilution, the mobility representing the combination of antibody with every available surface receptor. The development of an electrophoretic titration method is possible only if these maximum and minimum mobility values are well separated and if some portion of the curve between them has a slope sufficiently steep for practical experimental determinations. Therefore the first step is the determination of the curve using a potent antiserum so that the minimum mobility value can be measured directly or determined by reasonable extrapolation. Such a curve is given in Fig. 1. It is to be noted that at high concentrations of normal rabbit serum the mobility of the cells is also depressed.

Such curves serve to define several characteristics of the antigen-antibody system. From a comparison of such curves obtained for related systems it may be possible to detect more subtle differences between the systems than is possible using conventional titration procedures. Such detailed comparison will not be attempted at this time. If, however, it is assumed that various antisera differ only with respect to the concentration of antibody or antibodies present and not in their relative proportions, any characteristic property of the titration curve may be selected as the endpoint. The characteristic property which we have chosen is the concentration of the antiserum which produces a given mobility. For maximum sensitivity, this "endpoint" should be selected in the neighborhood of the inflection point of the curve. Applying this criterion to the data given in Fig. 1, the "electrophoretic titer" of sera prepared against whole cells or whole cell homogenates is defined as the reciprocal of the serum dilution which will cause homologous cells to assume an electrophoretic mobility of 1.5 μ/sec/volt/cm.

The assay of an antiserum of the type which the endpoint has been determined can be done very rapidly. As the excursion time corresponding to the endpoint under the given electrophoretic conditions is known, the change accompanying successive additions of antiserum can be followed by only a few observations until the endpoint is near. The titer can

TABLE I. Assay of Anti-lymphosarcoma Sera by Electrophoretic Method and Neutralization Test.

Antiserum No.	Neutralization test Serum dilution 1/2	1/4	1/8	1/16	1/32	1/64	Electrophoretic titer
Normal*	19/30	16/30	17/30	26/30	30/30		<50
4	2/6†	0/6	2/6	6/6	5/6	—	50
6	0/6	0/6	0/6	3/6	6/6	—	100
5b	0/6	0/6	0/6	0/6	4/6	—	200, 210
10	0/6	0/6	0/6	3/6	4/6	4/6	250
9	0/6	1/4	0/6	0/6	1/4	4/6	335, 335

* Summary of assay of 5 normal rabbit sera.

† $\frac{\text{No. of tumor takes}}{\text{No. of sites inoculated}}$.

be estimated from accurate determinations of the mobility of a few different dilutions in the neighborhood of the endpoint.

The available antisera which had been prepared against whole cells or whole cell homogenates were assayed. Table I summarizes the promising correlation between the "electrophoretic titer" and animal protection as demonstrated by the neutralization test(5).

It is obvious from the results of the two completely independent assays of antiserum No. 5b and antiserum No. 9 as well as from consideration of the titration curve that the electrophoretic titration is considerably more precise than our original criterion of acceptability.

Summary. 1. An electrophoretic technic is described which permits the assay of antisera for their power to combine with the cell surface, as distinguished from any reaction subsequent to combination. 2. This technic has been applied to cells of the Gardner lymphosarcoma 6-C3H-ED. 3. Preliminary studies with regard to the correlation of the electrophoretic titer and animal protection are promising.

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Peripheral Assimilation of Fructose in Man.* (20762)

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Uptake of fructose by hepatic cells has been demonstrated by a number of investigators(1-3). On the other hand, the ability of extrahepatic tissue, and specifically of muscle, to utilize fructose as such has been questioned(4). Other evidence suggests that fructose is utilized directly by muscle(5-7). To permit further study of the ability of peripheral tissues to assimilate fructose, successive peripheral arteriovenous fructose differences were measured in 11 human subjects during intravenous administration of fructose solutions.

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Methods. Eleven adult patients without known metabolic disorders served as subjects. All subjects were studied while they were at rest and in the postabsorptive state. In 9 subjects venous blood samples were obtained with a minimum of stasis from an antecubital vein, and almost simultaneously, capillary (arterial(8)) blood was collected by direct pipetting from a finger tip of the same extremity after cutaneous puncture. In 2 subjects arterial blood samples were obtained by means of a Cournand needle placed in a femoral artery, and venous blood was obtained through a cardiac-type catheter. The catheter was inserted in the basilic vein and manipulated under fluoroscopy so that its tip was in the distal portion of the subclavian vein. Roe's method(9) was used to measure fructose in whole blood and was found to be accurate within ±1.0 mg %. A Bowman constant infusion pump was used to deliver fructose into an arm vein at a constant controlled rate. First, 200 to 350 ml of 10% fructose in water solution was administered rapidly as a priming dose, and then constant fructose infusion was begun. The fructose always was injected at a rate of 0.58 g per minute. Blood samples were obtained at 15 ± 3 minute intervals (periods I-IV) for one hour beginning 30 to 100 minutes after start of fructose administration. One subject (E.G.) was given 875 ml of 10% fructose intravenously over an 80-minute period at 1.1 g per minute, and arterial and venous samples were obtained at 20-minute intervals during and after the infusion.

Results. Throughout the 4 periods, capillary (arterial) and venous fructose levels during constant fructose infusion remained virtually constant in individual subjects. For the 10 subjects who were given fructose at 0.58 g per minute, the average capillary fructose level for period I was 68.9 mg % (range 40-100); for period II, 67.6 mg % (range 39-98); for period III, 67.1 mg % (range 39-98); and for period IV, 66.1 mg % (range 37-88).

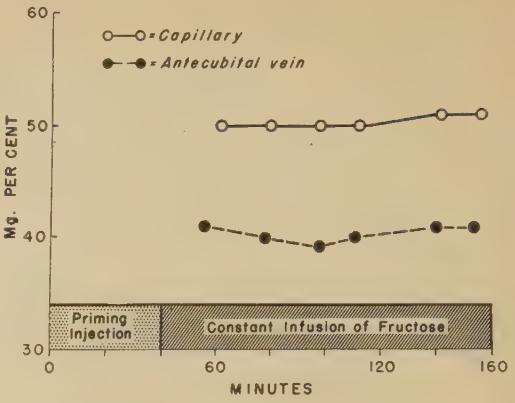

FIG. 1. Maintenance of constant capillary (arterial) fructose level and uniform capillary-venous fructose differences during constant, slow fructose infusion (0.58 g/min.).

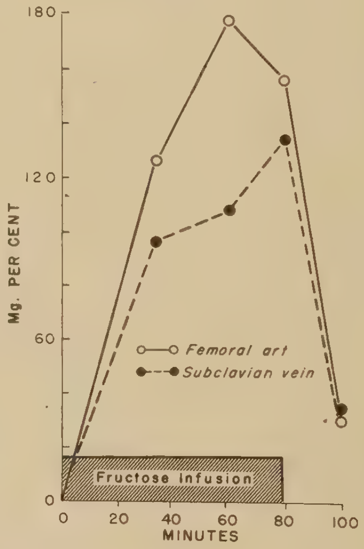

FIG. 2. Changing arterial fructose level and variable fructose arteriovenous differences during and after rapid fructose infusion (1.1 g/min.).

The average venous fructose level for period I was 57.3 mg % (range 30-78); for period II, 54.2 mg % (range 29-78); for period III, 55.5 mg % (range 26-78); and for period IV, 55.6 mg % (range 26-75).

The average arteriovenous fructose difference for period I was 11.6 ± 5.5 mg %; for period II, 13.4 ± 3.9 mg %; for period III, 11.7 ± 4.0 mg %; and for period IV, 10.6

± 3.1 mg %. A representative arteriovenous fructose curve during constant fructose infusion is shown in Fig. 1.

In patient E.G. (Fig. 2), arterial and venous fructose levels rose rapidly and fell precipitously after termination of the infusion. At one point, when the infusion was three-quarters completed, an arteriovenous fructose difference of 70 mg % was observed. However, at the end of the infusion this difference had decreased to 22 mg %. Twenty minutes after the end of the infusion the arterial fructose level had fallen to 32 mg % and the arteriovenous fructose difference was —4 mg %.

Discussion. In order to distinguish between peripheral arteriovenous differences due to simple diffusion into the interstitial space and those associated with tissue uptake it was found necessary to perform serial determinations of arterial and antecubital or subclavian venous fructose levels in subjects who were receiving fructose by constant slow infusion following administration of a priming dose. In this way it was possible to keep the arterial fructose level constant. An example of the difficulty involved in interpreting arteriovenous differences associated with a rapidly changing arterial fructose level is shown in Fig. 2, where the venous fructose level continues to rise even while the arterial level is falling off slightly.

It was believed that maintenance of appreciable fructose arteriovenous differences in the presence of constant arterial fructose levels could be regarded as good evidence for direct assimilation of the fructose by peripheral cells. Had the fructose arteriovenous differences quickly become negligible, or had the arterial and venous values for fructose shown a consistent trend in the direction of equilibrium(10), the findings could have been interpreted as evidence of simple diffusion rather than assimilation of the sugar. However, the results show that the arteriovenous fructose differences were well maintained in time.

Although these findings indicate that fructose enters peripheral cells, proof of utilization of this hexose by such cells would require technics other than those employed in this study.

Summary. 1. Serial peripheral arteriovenous fructose differences were measured in 10 human subjects receiving a constant intravenous infusion of 10% fructose in water, following an initial priming injection. Maintenance of consistent and appreciable arteriovenous differences under these circumstances occurred in every case, and this finding is interpreted as evidence for direct assimilation of fructose by the peripheral tissues. 2. The problem of distinguishing between diffusion, assimilation and utilization is discussed.

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Dihydroorotic Acid in Nutrition of Lactic Acid Bacteria. (20763)

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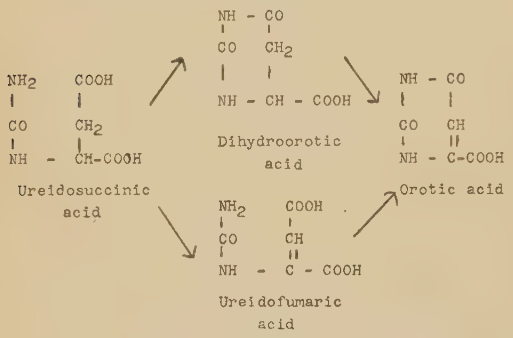
Lactobacillus bulgaricus 09 fails to grow on a semi-synthetic medium that supports excellent growth of related species(1,2). Good growth is obtained, however, in the presence of added orotic acid. Ureidosuccinic acid in considerably larger amounts may be substituted for orotic acid. Both are utilized for pyrimidine nucleotide and presumably, therefore, nucleic acid synthesis(3). If ureidosuccinic acid is an acylic precursor of the pyrimidine ring it can be visualized to yield orotic acid by one of two alternate pathways (a) cyclization to dihydroorotic acid followed by dehydrogenation to orotic acid, or, (b) dehydrogenation to ureidofumaric acid followed by cyclization to orotic acid.


NH₂ COOH CO CH₂ NH - CH-COOH
Ureidosuccinic acid
NH - CO CO CH₂ NH - CH - COOH
Dihydroorotic acid
NH - CO CO CH NH - C-COOH
Orotic acid
NH₂ COOH CO CH NH - C - COOH
Ureidofumaric acid

In an attempt at elucidating the mechanism involved, "dihydroorotic acid" was synthesized by the method of Bachstez and Cavallini(4) and tested for growth-promoting activity with *Lactobacillus bulgaricus* 09(5). The compound proved to be inactive in promoting growth. This finding was taken as evidence for the participation of ureidofumaric acid rather than dihydroorotic acid in the biosynthesis of orotic acid from ureidosuccinic acid. The hypothesis could not be tested, however, because of the unavailability of ureidofumaric acid. Lieberman and Kornberg recently have shown the existence in a cell-free preparation from an anaerobic bacterium of an enzyme that, in the presence of reduced diphosphopyridine nucleotide, reduces orotic acid. The product of the reduction was isolated and characterized by elementary composition and equivalent weight as dihydroorotic acid(6). Enzymatically derived dihydroorotic acid is different in a number of ways from the "dihydroorotic acid" of Bachstez and Cavallini and promotes growth of *Lactobacillus bulgaricus* 09(7). Dihydroorotic acid is asymetric about position 4 and consequently both D- and L- forms are possible theoretically. Both enantiomorphs of dihydroorotic acid as well as the racemate have now been synthesized in these laboratories by a new procedure and were made available for microbiological testing(8).

It is the purpose of this paper to show that (a) L-Dihydroorotic acid is utilized by some but not by all of a number of "pyrimidineless" lactobacilli (b) D-dihydroorotic acid in large amounts is an antimetabolite of L-dihydroorotic acid in pyrimidine utilization, and (c) differential microbiological procedures appear to be available for the qualitative and quantitative estimation of uracil, orotic acid, and dihydroorotic acid in a mixture of the three or in natural material free of interfering compounds.

Methods. The D-, L-, DL-dihydroorotic acids employed in this study were prepared as described by Miller *et al.*(8). The strains of lactobacilli and the media on which they were grown are summarized as follows: For *Lactobacillus arabinosus* ATCC 8014 the basal medium was composed of: 1 g norit-treated, acid-hydrolyzed, vitamin-free casein, 20 mg L-cystine, 40 mg DL-tryptophane, 1 mg each of adenine, guanine, and xanthine, 2 g sodium acetate, 4 g glucose, 1 ml each of salts A and B, 200 μg each of thiamin chloride, riboflavin, nicotinic acid, calcium pantothenate, 400 μg pyridoxine, 100 μg p-aminobenzoic acid, 1 μg biotin to a value of 100 ml of double-strength medium. The pH was 6.8. For

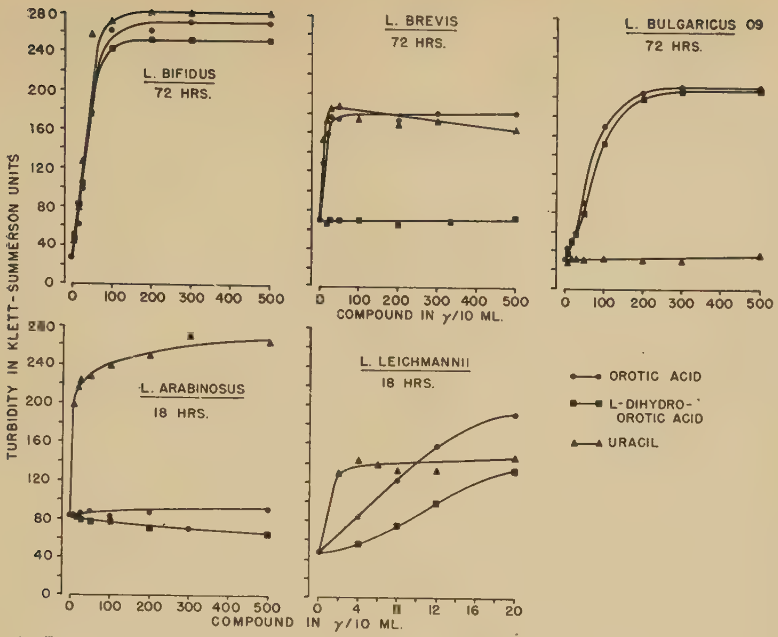


FIG. 1. Response of lactic acid bacteria to dihydroorotic acid and related compounds.

Lactobacillus leichmannii ATCC 4797 the basal medium was further supplemented with 100 mg DL-alanine, 20 mg guanylic acid, 0.2 ml tween 80, 100 mg thiomalic acid, 50 μg pyridoxal, 50 μg folic acid, and 0.002 μg vit. B_{12}. The pH was 6.8. For *Lactobacillus bulgaricus* 09 the medium was supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid, and 0.4 μg vit. B_{12}. The pH was 5.8. For *Lactobacillus brevis* ATCC 8287 the basal medium was further supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 0.2 ml tween 80, 50 μg pyridoxal, and 50 μg folic acid. The pH was 6.8. For *Lactobacillus helveticus* ATCC 335 the basal medium was supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 20 mg L-cysteine, 100 mg DL-alanine, 20 mg L-asparagine, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid, and 0.04 μg vit. B_{12}. The pH was 6.5. For *Lactobacillus bulgaricus* Sarles and *Lactobacillus bulgaricus* ATCC 8001 the basal medium was further supplemented with 1 g norit-treated, trypsin-digested, vitamin-free casein, 20 mg L-cysteine, 100 mg DL-alanine, 20 mg L-asparagine, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid, 0.04 μg vit. B_{12}, and 1650 units of pantetheine. The pH was 6.5. For *Lactobacillus bifidus* ATCC 4963 the basal medium was further supplemented with 500 mg norit-treated, trypsin-digested, vitamin-free casein, 0.2 ml tween 80, 50 μg pyridoxal, 50 μg folic acid and 2 μg vit. B_{12}. The pH was 6.8.

The lactobacilli studied vary with respect to the completeness of their pyrimidine requirement. For *L. bulgaricus* 09, *L. brevis, L. bulgaricus* 8001, *L. bulgaricus* Sarles, and *L. bifidus* a utilizable source of pyrimidine is *essential* for growth. For these species a prolonged incubation period of 72 hours was possible. Visual inspection of the tests showed that for these strains utilization of the various pyrimidine sources described in this paper is not influenced by the period of incubation up to 72 hours. For *L. arabinosus, L. leichmannii,* and *L. helveticus* a utilizable source of pyrimidine is only *stimulatory* for growth. With these species it was necessary to determine the amount of growth after a relatively short incubation period, usually of 18 hours. The effect of D-dihydroorotic acid on the utilization of orotic acid or L-dihydroorotic acid by *L. bulgaricus* 09 was studied in tests that

TABLE I. Effect of D-Dihydroorotic Acid on Growth of *Lactobacillus bulgaricus* 09.

Pyrimidine/tube		Turbidity*	
		24 hr	48 hr
0 γ orotic acid		0	0
10		22	20
20		39	34
30		54	52
40		60	69
60		70	102
100		81	150
20	+ 500 γ D-dihydroorotic acid	36	38
20	1000	19	42
20	1500	9	50
20	2500	10	41
50	+ 500	54	88
50	1000	28	102
50	1500	10	108
50	2500	7	51
0 γ L-dihydroorotic acid		0	0
10		24	16
20		38	31
30		49	50
40		57	65
60		51	93
100		62	143
20	+ 500 γ D-dihydroorotic acid	24	37
20	1000	9	40
20	1500	8	48
20	2500	4	32
50	+ 500	39	86
50	1000	19	96
50	1500	13	104
50	2500	6	31

* Corrected for color of medium.

were incubated for 24 hours and 48 hours. A Klett-Summerson photoelectric colorimeter was used to determine the extent of bacterial growth which is given in terms of arbitrary scale readings. Other non-specified procedures were as described previously(1,2) or according to customary technics with lactic acid bacteria.

Results. A summary of the data demonstrating the extent to which L-dihydroorotic acid can satisfy the pyrimidine requirement of certain lactobacilli is summarized in Fig. 1. For *L. bifidus*, *L. bulgaricus* Sarles and *L. bulgaricus* 8001 uracil, orotic acid, and L-dihydroorotic acid are equally effective. Data obtained with *L. bifidus* are given as an example. For *L. brevis* uracil and orotic acid are utilized and L-dihydroorotic acid is inactive. For *L. bulgaricus* 09 orotic acid or L-dihydroorotic acid satisfies the pyrimidine requirement but, as pointed out previously(1,2,9), uracil is inactive. For *L. arabinosus* and *L. helveticus* uracil alone is stimulatory with orotic acid and L- dihydroorotic acid essentially inactive. Data obtained with *L. arabinosus* are given as an example. The results with *L. leichmannii* are more difficult to interpret. At low levels of compound, uracil is more active followed by orotic acid and dihydroorotic acid in that order. At high levels orotic acid is more active than uracil.

D-dihydroorotic acid alone at a level up to 500 γ per tube (10 ml) is completely inactive in satisfying the pyrimidine requirement of *L. bulgaricus* 09. DL-Dihydroorotic acid is 50% as active as L-dihydroorotic acid or orotic acid. With an incubation period of 18-24 hours D-dihydroorotic acid in large amount is an antimetabolite of L-dihydroorotic acid and orotic acid (Table I). After 48 hours of incubation a large excess of D-dihydroorotic acid in the presence of L- dihydroorotic acid is utilized so that inhibition that was present during early growth disappears and growth occurs to an extent greater than that attributable only to the L-dihydroorotic acid present.

The data of Fig. 1 form the basis for possible microbiological methods of determining uracil, orotic acid, and dihydroorotic acid in a mixture of the three compounds. The uracil content would be given by the difference between the results obtained by *L. bifidus* assay and that obtained by *L. bulgaricus* 09 assay. The orotic acid content would be given by the difference between the result obtained by *L. brevis* assay and the value previously found for uracil. The dihydroorotic acid content would be given by the difference between the result obtained by *L. bifidus* and that obtained by *L. brevis*. Obviously other interfering compounds such as, for example, cytosine, thymine, and ureidosuccinic acid must be absent.

Summary. L-Dihydroorotic acid is utilized by a number of "pyrimidineless" lactobacilli to promote growth. D-Dihydroorotic acid in comparable amount is not utilized and under suitable conditions is an antimetabolite of L-dihydroorotic acid. Methods are outlined by which the microbiological determination of uracil, orotic acid or dihydroorotic acid in a mixture of the three is possible by differential assay.

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Isolation of West Nile Virus from Hooded Crow and Rock Pigeon in the Nile Delta.* (20764)

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(Introduced by J. Casals.)
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West Nile virus has repeatedly been isolated from the blood of febrile children(1-4) and from *Culex* mosquitoes(5) collected in an endemic area in the Nile Delta of Egypt. Because of the high incidence of immunity found in the human population during these studies, investigation was extended to the common indigenous birds of the area in an attempt to find potential warm-blooded virus circulators which might be serving as a virus source for widespread infection of mosquitoes during the peak transmission season. Serological survey of certain avian species indicated a high incidence of immunity to West Nile virus in domestic rock pigeons, *Columba livia,* and in wild hooded crows, *Corvus corone sardonius.* Experimental infection by the feeding of infected *Culex* mosquitoes on crows showed that this species circulated West Nile virus in high titer for at least 5 days(6).

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The opinions or assertions contained herein are the private ones of the authors, and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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Considerable attention was directed to examination of these birds as collected in their natural habitat beginning early in June 1953. This report deals with the isolation of West Nile virus from two pigeons and a crow.

Materials and methods. Blood for examination was routinely drawn by cardiac puncture into a sterile 2cc or 10cc syringe. Captured birds were bled by needle puncture through intact skin. In specimens obtained by shotgun collection, the chest was incised with a scalpel, exposing the base of the heart. Blood was drawn directly through a 21-gauge needle inserted into the cardiac chambers or great vessels. Blood samples were emptied into sterile 13 x 100 mm or 12 x 75 mm test tubes closed with sterile rubber stoppers. These tubes were placed on ice in a wide-mouth thermos for transport to the laboratory, where they arrived within two to three hours after collection. In the laboratory the clot was broken up and the specimen centrifuged at 1500 rpm for 10 minutes. The serum was drawn off by sterile pipette, cultured on blood agar, and deposited in a numbered screw-top glass vial for reference storage at –20°C. A portion of each serum was inoculated, .02 ml intracerebrally and .03 ml subcutaneously, into each of 7 two- or three-day-old white Swiss mice. The few specimens contaminated by bacteria were filtered through Seitz apparatus and reinoculated. The mice were observed daily for signs of sickness, paralysis, or death. When abnormal signs appeared, the mouse was sacrificed. The brain harvest was triturated into a 10% suspension in 10% inactivated normal rabbit serum. This material was passed as above into a group of infant mice, and .03 ml intracerebrally into a group of 6 three-week-old mice. When any strain of virus produced paralysis and death consistently in the entire group in four to seven days, the brain harvest was titrated in a neutralization test with West Nile-immune and nonimmune serum. Observation of characteristic paralysis and death in three-week-old mice and neutralization of more than 100 LD_{50} of the virus strain by West Nile-immune serum were considered adequate evidence that the virus was West Nile.

Results. On July 5, 1953, one of us (HSH), on a visit to Chenuda Farm 15 kilometers east of Kafr el Sheikh in the north central area of the Nile Delta, had his attention called by the farm owner to a sick pigeon, *Columba livia.* It was a young bird, fully feathered and estimated to be 3 to 4 weeks old. Although of flying age it was too weak even to walk. No paralysis or tremor was noted at the time. The bird (AN-1230) was received alive in our laboratory on July 6. It was exsanguinated by cardiac puncture, and 10% suspensions of brain and spleen in 10% normal rabbit serum were prepared. These suspensions and the serum were each inoculated into a group of infant mice. The mice showed characteristic paralysis on the fifth postinoculation day and all were dead on the sixth. A 10% suspension of brain harvest was passed into infant and 3-week-old mice; paralysis and death, characteristic of West Nile virus infection, occurred on the fourth and fifth days. A 10% suspension of brain harvest material from these mice was titrated against nonimmune and West Nile-immune baboon sera which showed a 2.89 log difference. It was therefore concluded that this strain was a West Nile virus.

Beginning early in June 1953, periodic collections of 6 to 15 specimens of the hooded crow, *Corvus corone sardonius,* were made at 5- to 14-day intervals in the environs of Sindbis village about 30 kilometers north of Cairo. A specimen (AN-1246) of serum obtained on July 14, 1953, and prepared by the methods described previously, was inoculated into a group of 7 infant mice. One mouse died on the first day; the remaining 6 died on the sixth postinoculation day. A Seitz-filtered 10% suspension of two brains harvested was passed intracerebrally into 3-day-old mice. These were paralyzed and died on the sixth and seventh postinoculation days. Brain harvest material from these mice, passed intracerebrally into 7 two-day-old and 6 three-week-old mice, produced paralysis and death on the fourth and fifth postinoculation days in all animals. Brain harvest material was titrated against nonimmune and West Nile-immune sera of the same baboon and 3.5 logs of difference resulted. It was concluded that this strain also was a West Nile virus.

In following up the isolation of West Nile virus from the sick pigeon (AN-1230), a return was made to Chenuda Farm on August 6, 1953, when 33 apparently healthy pigeons were captured there and 2 cc of blood was collected by cardiac puncture before the birds were released. Serum from all these specimens was inoculated into one-day-old mice for attempted virus isolation according to the procedure described. On the seventh postinoculation day one mouse was found with characteristic paralysis. The animal was sacrificed and a 10% brain suspension was passed into a group of 2-day-old mice. All became paralyzed and died on the third and fourth postinoculation days. Brain harvest material from these mice was passed in a 10% suspension into 3-day-old and 3-week-old mice. All developed paralysis and died by the fifth postinoculation day.

Brain harvest material was titrated against nonimmune and West Nile-immune sera of the same baboon and a difference of 2.4 logs specific neutralization was calculated. This was taken as adequate evidence that the strain isolated was an additional West Nile virus.

The failure of 6 of the 7 mice initially inoculated with the pigeon serum to show paralysis or death may have been due to low titer of virus in the serum. It should also be noted that this same serum (AN-1324) neutralized 100 LD_{50} of West Nile virus in a routine neutralization test using 33rd passage strain of West Nile virus originally isolated by Smithburn *et al.* in Uganda(7).

Discussion. Eastern equine encephalitis virus has repeatedly been isolated in North America from naturally infected avian hosts; *i.e.*, from the brains of ring-necked pheasants (8-11) and domestic pigeons(12) and the blood of a purple grackle(13). Western equine encephalitis virus has been recovered from the brain and spleen of a prairie chicken (14) and the blood of a nestling magpie and nestling redwing blackbirds(15).

Studies of western equine and St. Louis viruses in regard to isolation from wild-caught mosquitoes, precipitin testing of mosquito stomach contents, neutralizing antibody surveys of wild and domestic avian species, and experimental infection of some species have rather firmly established the potential role of certain avian hosts in the epidemiology of these encephalitides(16-19).

The immunological relationship of West Nile to Japanese B and St. Louis encephalitis viruses has repeatedly been shown(20-23). The incidence of positive neutralization of 100 LD_{50} of West Nile virus by the sera of pigeons and crows, as well as of human residents, in areas where the virus was recovered from bird blood is 50% or more. It is of some significance therefore that West Nile virus has been isolated from birds in nature in an endemic area of West Nile virus infection in the Nile Delta of Egypt.

The isolations of these strains from birds in July and early August coincided with the period during which most West Nile virus isolations were made from *Culex* mosquitoes in 1952 and 1953. Over 400 acres contiguous with the location of the pigeon loft were planted in well-watered rice, which provided breeding ponds for large numbers of *Culex* mosquitoes.

The pigeons from which the isolations were made were a feral type deliberately attracted to large cone-shaped lofts in order to produce guano for use as fertilizer on the farm. The smaller sleek form of the feral type distinguishes it from the domestic permanent resident pigeons which are raised for food by the fellahin.

The Chenuda farm owner stated that epidemics of illness such as were manifested by the sick pigeon had occurred in previous years. He observed that during these epidemics the apparently healthy birds deserted the loft to move to a new and distant locality. Several months passed before another feral flock took up residence in the deserted loft. This presents an interesting possible mechanism for dissemination of the virus by local pigeon movements.

A cursory survey of nesting hooded crows in the Sindbis area indicates that this is the third commonest breeding species and may contribute as many as 75 nonimmune juveniles per square kilometer each year.

Finding circulating West Nile virus in birds commonly resident in endemic areas of human infection in Egypt adds further support to the concept that avian species play an important if not essential role in the transmission of certain of the arthropod-borne virus encephalitides.

Summary. 1. Strains of West Nile virus were isolated from the blood of two pigeons, *Columba livia,* and one hooded crow, *Corvus corone sardonius,* in an endemic area of the Nile Delta of Egypt in July and August 1953. The virus was also obtained from the brain and spleen of one of the pigeons. 2. The relation of these isolations to the environment is briefly discussed.

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Preparation of S,S′-Dibenzyloxytocin and Its Reconversion to Oxytocin.* (20765)

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In previous communications evidence has been presented that oxytocin is a polypeptide consisting of cystine and 7 other amino acids plus 3 moles of ammonia(1,2). It was further shown that the disulfide linkage was unquestionably present in some form of cyclic structure since no evidence for the presence of more than one moiety was found after cleavage of the disulfide by means of oxidation with performic acid(3) or removal of the sulfur by Raney nickel(4). A study of the purified performic acid-oxidized oxytocin revealed that the oxidized material possessed 2 cysteic acid residues, one of the moles of cysteic acid occupying a terminal position with its amino group free(5). From these results it would be expected that reduction of the disulfide to a sulfhydryl form followed by benzylation should result in a benzylated oxytocin containing 2 *S*-benzylcysteine residues and having a structure analogous to the performic acid-oxidized material if no other reactions occurred in the formation of the respective derivatives. It may be recalled that in 1935 Sealock and du Vigneaud(6) had shown that treatment of partially purified oxytocin with cysteine in aqueous solution at pH 7.5 at room temperature did not result in inactivation. Treatment of the resulting solution with benzyl chloride inactivated the hormone, whereas treatment of the original oxytocin preparation under the same conditions did not result in inactivation. It was predicted from this behavior that oxytocin contained sulfur in the form of a disulfide, and that the reduced or sulfhydryl form of the hormone was also active.

Preliminary studies with partially purified oxytocin in these laboratories has also shown that oxytocin could be reduced in liquid ammonia with metallic sodium without appreciable loss of activity and further that addition of benzyl chloride to the liquid ammonia reaction mixture resulted in inactivation. With highly purified oxytocin available, it was felt that reduction and benzylation in liquid ammonia should be tried on such material in the hope that a benzylated oxytocin might be separated in reasonably pure form and that through quantitative amino acid analysis one would be able to ascertain whether the composition agreed with what might be expected.

229 mg of oxytocin, with a potency of approximately 500 units/mg and with a typical amino acid composition(7), were benzylated in batches of approximately 50-70 mg. The benzylation was carried out as follows: 50 mg of oxytocin were placed in the reaction vessel, dissolved in 15 ml of ammonia, and sodium was added until a lingering blue color was obtained. 0.1 ml of benzyl chloride was added and the solution was stirred for 20 minutes. Before removal of the ammonia, a few crystals of ammonium iodide were added. The dry residue was extracted with small volumes of anhydrous acetone, the residual acetone was removed *in vacuo*, the residue was extracted with small volumes of cold 2% acetic acid, and the product was dried *in vacuo* over P_2O_5 at room temperature. From 229 mg of oxytocin, 188.3 mg of benzylated oxytocin were obtained. A sample of this material gave a negative nitroprusside test, which remained negative on the addition of cyanide, whereas a sample of oxytocin gave a positive nitroprusside test

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* A preliminary announcement of these results was made recently (du Vigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., Katsoyannis, P. G., and Gordon, S., *J. Am. Chem. Soc.*, 1953, v75, 4879.)

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after the addition of cyanide. A sample of the benzylated oxytocin was analyzed for its constituent amino acids by the starch column technic(8,9) and found to contain 2 moles of *S*-benzylcysteine, one mole each of leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and a trace of cystine. The solvent system used by Stein and Moore (9) for the resolution of leucine and isoleucine was found when slightly modified to allow the determination of *S*-benzylcysteine in addition to leucine and isoleucine. When the solvent system 1:1:0.27 *n*-butyl alcohol-benzyl alcohol-water was used, an authentic sample of *S*-benzylcysteine was found to emerge at effluent ml 15 and to possess a color yield of 0.92. Assay of a portion of the benzylated oxytocin after solution in one drop of glacial acetic acid and dilution with water indicated it to be inactive.

From these studies it is obvious that the sulfhydryl form of oxytocin is biologically active and that when the sulfhydryl groups are covered by benzyl groups the resulting compound is inactive. Whether the sulfhydryl form *in vivo* is active *per se* or is converted to a disulfide form is an open question at the present time. On the other hand, it is possible that the hormone might have to be reduced in the tissues to exert its biological effect. Earlier attempts to regenerate active material from the benzylated oxytocin by debenzylation in our laboratories had been negative. It was obvious, however, that if debenzylation could be brought about to reactivate the material and if on oxidation the original oxytocin could be obtained with the disulfide in the cyclic structure, an approach to the synthesis of oxytocin was feasible once the sequence of the amino acids in the *S*-benzylated oxytocin or the performic acid-oxidized oxytocin had been established. Thus, an eventual synthesis of oxytocin might be possible through the application of the method of cystine peptide synthesis introduced by du Vigneaud and Miller(10), in which the sulfhydryl group of cysteine is protected by a benzyl group during a series of reactions and the benzyl group is removed at the end of the series by sodium in liquid ammonia. This method was based on the earlier findings that a sulfhydryl compound could be benzylated in liquid ammonia(11) and that the resulting benzyl thio ether could be cleaved by sodium in liquid ammonia(12). A study of the debenzylation of the inactivated benzylated oxytocin was therefore undertaken. This has led to the regeneration of what we believe to be oxytocin.

Approximately 20 mg of benzylated oxytocin were dissolved in 20 ml of ammonia and reduced with sodium. A few crystals of ammonium chloride were added to discharge the slight blue color and the ammonia was removed *in vacuo*. The dry residue was quickly dissolved in 100 ml of cold 0.005% acetic acid and the pH of the solution was adjusted to 7-7.5. The solution was aerated with CO_2-free air for one hour and then concentrated to a small volume in a rotary evaporator(13) at a temperature below 35° and lyophilized. Assays of the solution indicated the regeneration of activity. Several batches of the regenerated material were combined. From a total of 160 mg of benzylated oxytocin approximately 12,700 units of activity were obtained. This represented a yield of approximately 20% on the basis of the activity that might be theoretically derived from this amount of benzylated oxytocin. The regenerated material was subjected to countercurrent distribution(14) with the solvent system *sec*-butyl alcohol-0.05% acetic acid. One hundred transfers were applied and the distribution was analyzed by weight determination. From the results it was quite evident that complete separation of the salt peak and the activity peak had not taken place. Ultraviolet absorption at 275 mμ was also used to analyze the distribution. A maximum at approximately Tube 27 coincided with the peak containing the active principle found by the weight analysis. The partition coefficient was found to be approximately 0.37. The distribution was then continued for another 100 transfers. An assay of one of the peak tubes indicated an activity of 410 U.S.P. units per mg by the blood pressure method. The material had the expected activity on the isolated uterine strip. Tubes 43-60 inclusive were combined, concentrated, and lyophilized to yield 28.5 mg of regenerated, active material which was used to establish the nature of the regenerated material.

A sample of the regenerated active material was analyzed for constituent amino acids by the starch column method(8,9). It was found to have the same amino acids as the original oxytocin in essentially 1:1 ratio and *S*-benzylcysteine was found to be absent. Electrophoretic studies at pH 9.7 (glycine buffer) and pH 4.5 (acetate buffer) were carried out by the method described by Kunkel, Taylor, and du Vigneaud(15). Fifty μg of regenerated oxytocic material and the same amount of original oxytocin were applied to Whatman No. 3 MM filter paper and 300 volts were applied for 2 hours. The regenerated material behaved like the original oxytocin; however, in the case of the acetate buffer, a trace of a second component was noted. Crystallization of regenerated material as the flavianate was accomplished and the crystals were compared with the flavianate of the original oxytocin(7). No difference in melting point, solubility, or crystalline structure could be observed between the crystalline flavianate of the regenerated material and the crystalline flavianate of the original oxytocin. Optical rotations of both regenerated and original oxytocin were determined. For the regenerated oxytocin, the rotation was $[\alpha]_D^{22} = -27.9°$ (0.57% in water) and for the original oxytocin, the value was $[\alpha]_D^{22} = -26.2°$ (0.53% in water).

Although the activity of the regenerated material was not quite as high as that of the original oxytocin, we believe that the data obtained on the properties of the regenerated oxytocic material warrant the conclusion that oxytocin has been regenerated from the S,S′-dibenzyl derivative by debenzylation followed by oxidation.

Summary. The benzylation of oxytocin by reduction with metallic sodium in liquid ammonia and subsequent treatment with benzyl chloride has been found to result in the formation of an S,S′-dibenzyl derivative which is biologically inactive. The activity has been regenerated by reduction of the benzylated oxytocin with sodium in liquid ammonia and a comparison of the material after oxidation with oxytocin itself has led to the conclusion that the biologically active material so formed represents oxytocin.

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Isoniazid Distribution in Body Fluids of the Dog and the Rabbit.[*] (20766)

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Since the advent of isonicotinic acid hydrazide isoniazid as an agent in the treatment of tuberculosis(1,2), efforts have been directed at determining the absorption and distribution of the drug in body tissues and fluids(3-8). A series of determinations are reported here which further elucidate the distribution of isoniazid in body fluids.

TABLE I. Isoniazid Concentrations in Maternal and Fetal Plasma, and Amniotic Fluid of 5 Rabbits, All Expressed in μg per ml.

Hr after i.v. admin.	Maternal plasma	Fetal plasma	Amniotic fluid
2	4.9	5.2	
3	2.3	5.2	
2	6.2	6.8	
3	5.4	5.9	
2	4.8		5.5
3	3.0		5.5
2	5.2		3.5
3	5.0		5.2
2	4.6		5.5
3	4.4		5.1

TABLE II. Isoniazid Concentrations in Plasma and Milk of the 3 Lactating Dogs, All Expressed in μg per ml.

Hr after oral admin.	Plasma	Milk
1	15.7	16.5
2	17.8	31.2
4	8.4	16.4
2	18.2	17.6
4	10.6	15.6

Experimental. Five pregnant rabbits were anesthetized with 40 mg per kg of 'Amytal Sodium' (Amobarbital Sodium, Lilly) intravenously, about 2 hours after intravenous administration of 10 mg per kg of isoniazid. The body cavity was then opened and 2 or 3 intact loculi removed. After one hour, the remaining loculi were detached. Amniotic fluid free from blood contamination was withdrawn with a hypodermic needle and syringe from the intact placentae. Fetal blood was secured by cardiac puncture, and pooled. Maternal blood was taken from the medial artery of the ear or by cardiac puncture. To secure sufficient volume for accurate isoniazid determination amniotic fluid was obtained from 20-22-day pregnant animals, and the fetal blood from 28-30-day-old fetuses. In the dog experiments isoniazid was given orally by stomach tube in a dose of 20 mg per kg. Blood samples were taken from the jugular vein. Three lactating dogs were milked dry manually before, and at intervals after, drug administration. Isoniazid was given to 5 other dogs before phenobarbital anesthesia. The pancreatic and left submaxillary ducts were cannulated. The samples were collected for 15 minutes at the beginning of each hour. Prior to each collection period secretions were stimulated by the intravenous administration of secretin and pilocarpine. The common bile duct and the ureters of 3 of the dogs were also cannulated. Bile and urine were collected continuously throughout the assay period. Two unanesthetized female dogs with ligated cystic ducts and duodenal fistulas(9) so arranged that plastic tubing could be inserted into the common bile duct were used for an excretion study. Bile was collected continuously for six hours with the dog resting on its side. Urine was obtained by catheterization.

The analysis of the isoniazid content of the various body fluids was performed according to the technic of Kelly and Poet(10). This procedure relies on extraction of isoniazid from biological material with an ether-isoamyl alcohol mixture, redistribution in 0.1 N HCl and subsequent color formation with *p*-dimethylaminobenzyaldehyde. Photometric readings were made with a Beckman model DU spectrophotometer at 450 mμ. All blood was heparinized. Plasma samples exhibiting hemolysis gave unreliable results and were discarded. Bile, when analyzed by this procedure, produced a cloudiness in the color reaction. This was avoided by using small

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[*] The authors are deeply indebted to Drs. F. G. Henderson and C. C. Lee for their invaluable assistance in this study.

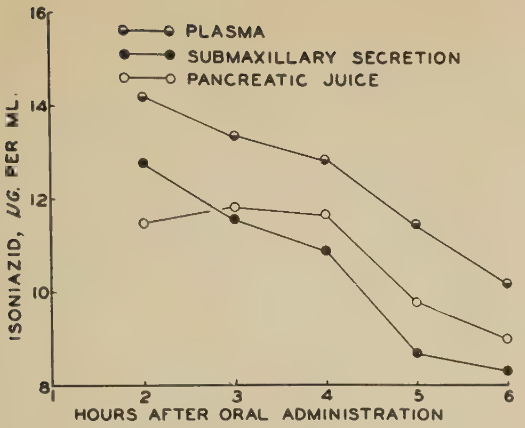

FIG. 1. Average isoniazid concentrations in the plasma, submaxillary secretion, and pancreatic juice of 5 dogs receiving 20 mg/kg orally.

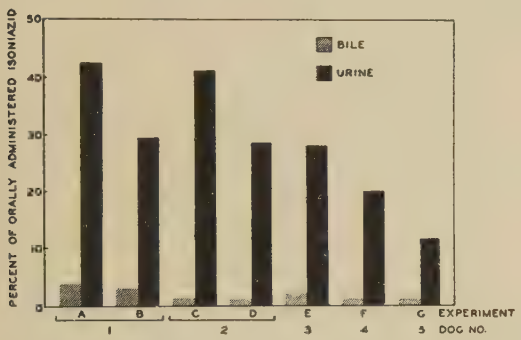

FIG. 2. Isoniazid excretion for the first 6 hr following oral administration of 20 mg/kg in the dog. Numbers 1 and 2 were unanesthetized while 3, 4 and 5 were anesthetized with phenobarbital.

volumes (.02-.04 ml). The method yielded reliable results only when bile isoniazid concentrations exceeded 25 µg per ml. No difficulty was encountered with the other fluids analyzed.

Results. A study was performed in the pregnant rabbit to detect whether isoniazid is transferred from the maternal circulation to the fetus and the amniotic fluid. Results as shown in Table I demonstrate that isoniazid readily passes through the placental barrier.

In 3 of the 5 milk samples taken from 3 dogs, the isoniazid concentration exceeded that of the circulating plasma and was equivalent in the other 2 (Table II).

The isoniazid levels in submaxillary secretion and pancreatic juice approximated the plasma levels throughout the period of 2 to 6 hours after oral administration as shown in Fig. 1. The disappearance rate also closely followed that of the plasma in both instances.

Biliary and urinary excretion was followed in 3 anesthetized and 2 unanesthetized dogs for 6 hours subsequent to oral administration of isoniazid. Total recovery in the bile ranged from 0.9 to 3.6% of the dose during the 6 hours. Urine excretion varied from 12.0 to 42.5% in the same dogs (Fig. 2). In 4 experiments with the unanesthetized dogs, the bile recovery varied from 3.5 to 9.2% of the total combined urinary and biliary excretion. In the 3 anesthetized dogs the bile recovery varied from 5.8 to 8.5% of the total. Since it appeared that anesthesia produced no preferential effect on either the biliary or urinary excretion of the drug, the mean rates of excretion were computed. As is shown in Fig. 3, the mean maximal excretion rate for both urine and bile was reached simultaneously at the second hour. Diminution of the rates occurred nearly proportionately.

Discussion. The dose levels of isoniazid employed in our experimental animals were higher than those in clinical usage. High concentrations in body fluids enhanced the accuracy of the chemical determinations, particularly in the case of the bile. No toxic symptoms were noted in any of these acute experiments. The small number of animals used make conclusions valid only in so far as

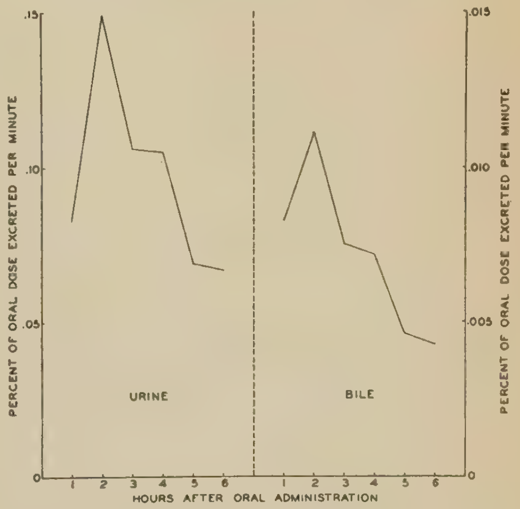

FIG. 3. Average urinary and biliary excretion rate of isoniazid in 7 experiments with 5 dogs receiving 20 mg/kg orally.

trends of isoniazid distribution and excretion are concerned.

Rubin *et al.*(6) found that in the dog the spinal fluid and most tissue concentrations follow plasma levels closely. Our data are in general agreement with theirs in that the isoniazid concentration in the pancreatic juice and the submaxillary secretion of the dog, as well as the fetal plasma and the amniotic fluid of the rabbit, also correspond to plasma levels. Camurri(7) reported a similar finding in the fetal plasma of the rabbit, but found the amniotic fluid to have a greater isoniazid concentration than the maternal plasma.

We found the isoniazid levels in the milk of the dog to be higher than in the plasma in 3 out of 5 instances. Camurri(8), on the other hand, reported isoniazid levels in human milk as significantly lower than plasma. This might be due to a species difference. Certainly further work is necessary to establish the role of the mammary gland in isoniazid excretion.

The peak urinary excretion of isoniazid in the dog was reported by Rubin *et al.*(6) to occur between the second and fourth hour aftei oral administration. This was confirmed by us in both anesthetized and unanesthetized dogs. The biliary excretion reached its peak at the same time as the urinary excretion, but at less than one-tenth the magnitude.

Summary. 1. In the rabbit isoniazid was found to pass freely through the placental barrier. 2. In the unanesthetized dog isoniazid appeared in the milk at concentrations equal to or greater than those of the plasma. 3. In the phenobarbitalized dog the concentrations of isoniazid in the submaxillary secretion and the pancreatic juice were found to be at near plasma levels. 4. In both anesthetized and unanesthetized dogs, total isoniazid recovered from urine and bile at the sixth hour following oral administration varied from 12.9 to 46.0%. Biliary recovery was less than one-tenth of the total in the 5 dogs studied.

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